IRON ACQUISITION IN *LISTERIA MONOCYTOGENES*

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by

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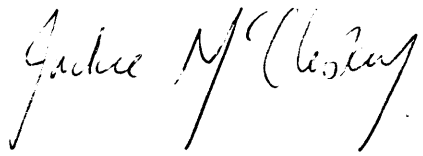
DECLARATION

This thesis is the original work of the author:



Jackie McCluskey

DEDICATION

For my Nana & Granda

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ABSTRACT

Jackie McCluskey: Iron Acquisition in *Listeria monocytogenes*

This investigation was designed to study iron acquisition mechanisms of *Listeria monocytogenes*. Molecular biology techniques were employed.

A listerial genomic library in *E. coli* and a library of listerial transposon mutants was constructed and screened. The assay used to screen both libraries was based on the ability of a ferrous iron chelator, ferrozine, to produce a red colour when complexed with ferrous iron. After screening approximately 10,000 clones from the genomic library and 12,000 mutants from the transposon library of mutants, 11 apparently ferric reductase-negative transposon mutants were identified. Subsequent analysis of these mutants showed them to be ferric reductase-positive. The reason for this reversal of phenotype is not known and was not investigated further.

To identify genes which were involved in the growth of *L. monocytogenes* in low-iron environments, the library of listerial transposon mutants was screened in low-iron medium. From the 5,500 screened, two mutants were identified which showed reduced ability to grow in both low-ferric and low-ferrous iron medium. In the high-iron medium, the growth of the mutants was similar to that of the wild type.

Mutant 9E was phenotypically similar to the wild type except for the inability to grow in low-iron medium. Mutant 7D had two additional difference; increased haemolytic activity and decreased motility when observed using phase microscopy. Sequencing of the flanking chromosomal DNA led to the identity of the genes which had been disrupted; *fla*A in mutant 7D and *arg*C in mutant 9E. Sequence analysis upstream from the *fla*A gene identified a new gene, *che*V, in *L. monocytogenes*. The relationship of these mutations to the observed phenotypes was discussed. The ability of both mutants to grow *in vivo* was similar to that of the parental wild type.

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INTRODUCTION

1.1 *LISTERIA MONOCYTOGENES* - HISTORICAL INTRODUCTION

Listeria monocytogenes is a member of the genus *Listeria* which currently consists of six species, *L. monocytogenes, L. innocua, L. ivanovii, L. seeligeri, L. welshimeri and L. grayi* (Stuart & Welshimer, 1974; Feresu & Jones, 1988). The species *L. monocytogenes* and *L. ivanovii* have been recognised as important pathogens of animals and humans since early in the history of medical bacteriology (Gray & Killinger, 1966). They cause clinical conditions varying from mild influenza-like illnesses to meningitis and meningoencephalitis, all of which are covered by the term listeriosis.

Listeria monocytogenes was first isolated and characterised by Murray, Webb and Swan in 1926. These workers at Cambridge University, in 1924, were able to show this organism as the causative agent of septicaemic infection of laboratory animals such as guinea pigs and rabbits and they named the organism *Bacterium monocytogenes*. The genus *Bacterium* was chosen because Murray *et al.* were not exactly sure into which genus this organism should be classified, while the specific name *monocytogenes* was assigned due to the characteristic monocytosis observed following infection with this organism. Although Murray *et al.* (1926) are generally credited with the first accurate description of *L. monocytogenes*, other reports suggest that the organism was isolated from similar infections prior to 1924 (Atkinson, 1917; Dick, 1920).

Following the works of Murray *et al.* (1926), Pirie described an organism which he had isolated from gerbils and which he named *Listerella hepatolytica* in honour of the British microbiologist Lord Lister (Pirie, 1927). This was later recognised by Pirie as being identical to the organism isolated by Murray *et al.* and the name was changed to *Listerella monocytogenes* (Addendum Pirie, 1927). The name change from *Listerella monocytogenes* to *Listeria monocytogenes* took place in 1940 because the genus name

Listerella had been used previously for a mycetozoan (Pirie, 1940). This new name was included in the 6th edition of Bergey's Manual of Determinative Bacteriology.

1.2 NATURE OF THE PATHOGEN

1.2.1 Bacteriological characteristics

The principal characteristics of *L. monocytogenes* and all the members of the genus are: Gram positive, non-spore forming, non-acid-fast, pleomorphic rod shaped bacteria with round ends (Gray, 1960). When observed microscopically, fresh isolates that produce colonies with smooth edges appear as short diptheroid-like rods approximately 1.0-2.0 μm x 0.5 μm, while bacteria from colonies with rough edges appear as longer rods measuring 6-20 μm in length and often form filaments (Gray, 1960). The variation in colonial and bacterial morphology is thought to be correlated with the amount of a protein, p60, produced by the bacterium (Kuhn & Goebel, 1989). Smooth form isolates produce more p60 than isolates of the rough form. In addition to these typical morphological characteristics, *L. monocytogenes* often exhibits a tumbling motility due to flagellar rotation, which is a useful characteristic in identifying the organisms (Seeliger, 1961). The presence of flagella is dependent on the temperature which the bacteria were grown prior to examination of the flagella (Galsworthy *et al.*, 1990; Kathariou *et al.*, 1995). Flagella are more commonly found on bacteria grown at 22°C than on those grown at 37°C and consequently, bacteria grown at the former temperature are more motile (Galsworthy *et al.*, 1990).

Listeria monocytogenes is capable of growing at temperatures between 1 and 45°C with optimum growth occurring in temperatures between 30 and 37°C (Seeliger & Jones, 1986). The organism can be cultured in a number of different bacteriological media

(Gray, 1960; Seeliger, 1961; Ralovich, 1984) although Tryptose Agar is generally used for maintaining stocks and for subsequent culturing within the laboratory (Gray, 1960). The purity of a culture grown on a clear medium such as Tryptose Agar can be examined under magnification using obliquely transmitted light (Henry, 1933), as colonies of *L. monocytogenes* appear bluish-green and have a finely textured surface when illuminated in this manner.

Listeria monocytogenes was reported as being able to grow at pH values between 5.6-9.6 (Seeliger & Jones, 1986) with maximum growth occurring at neutral to slightly alkaline pH values. More recent studies have shown the ability of the organism to grow at pH values as low as 4.4-4.6 (Farber *et al.*, 1989). Oxygen levels also influence the growth of the organism and reports by Seeliger (1961) and Ryser & Marth (1991) indicated that growth is enhanced in conditions where the oxygen tension is reduced compared with air.

1.2.2 Taxonomy

Until the 7th edition of Bergeys Manual of Determinative Bacteriology (Breed *et al.*, 1948, 1957) the genus *Listeria* was monospecific, containing only *L. monocytogenes*, and was included in the family of *Corynebacteriacae*. However in the 8th edition of Bergey's Manual of Determinative Bacteriology the genus *Listeria* was listed alongside the genera *Erysipelothrix* and *Caryophanon* in the "genera of uncertain affiliation" (Buchanan & Gibbons 1974). This alteration was based on the findings of various studies on different aspects of the genus, such as cell wall composition, nucleic acids and numerical taxonomy (Schleifer & Kandler, 1971; Stuart & Welshimer, 1974; Feresu & Jones, 1988). More recent findings, based on 16S rRNA reverse transcriptase analysis, suggested that the genus *Listeria* be removed from the current family and assigned to a

new family *Listeriaceae* which would include the genera *Listeria* and *Brochothrix* (Collins *et al.*, 1991).

In recent years a number of amendments have occurred within the genus. In 1985, eight species were included in the genus *Listeria*, these being *L. monocytogenes, L. innocua, L. ivanovii, L. seeligeri, L. welshimeri, L. denitrificans, L. murrayi and L. grayi* (Audurier *et al.*, 1984). Although these eight species can be found in the 9th edition of Bergey's Manual of Systematic Bacteriology, three of these species - namely *L. denitrificans*, *L. murrayi* and *L. grayi* had been categorised as species *incertae sedis* (Seeliger & Jones, 1986). The basis of these alterations was the results obtained from molecular biological, numerical taxonomic and chemical studies of these species. The classification of *L. murrayi* and *L. grayi* was altered because numerical taxonomic and chemical studies indicated a very close relationship between both species. Rocourt *et al.* (1987b) showed the close relationship of *L. murrayi* to *L. monocytogenes* using 16S r RNA cataloguing, while also showing the very close relationship of *L. murrayi* and *L. grayi* using DNA/DNA hybridisation (Rocourt *et al.*, 1987a). Other workers have suggested that *L. murrayi* and *L. grayi* are not in fact distinct species but rather should be members of the one species, namely *L. grayi* (Feresu & Jones, 1988; Collins *et al.*, 1991; Jones *et al.*, 1990; Rocourt *et al.*, 1992).

Listeria denitrificans was shown not to belong to the genus *Listeria* by a number of workers, all of whom reached their conclusion following numerical taxonomic, biochemical, morphological or nucleic acid studies (Collins *et al.*, 1983; Espaze *et al.*, 1986; Fiedler & Seger, 1983; Fiedler *et al.*, 1984; Jones, 1975; Jones *et al.*, 1986; Stuart and Pease, 1972; Stuart & Welshimer, 1973,1974; Wilkinson and Jones, 1977). Rocourt *et al.* (1987a) proposed this species be reclassified in a new genus *Jonesia*, as *Jonesia denitrificans*.

The remaining five species *L. monocytogenes, L. innocua, L. ivanovii, L. seeligeri* and *L. welshimeri* have been shown to have a very high degree of DNA homology (Rocourt *et al.*, 1982) and also many similarities in phenotypic and biochemical characteristics (Seeliger & Jones, 1988). Multilocus enzyme electrophoresis was used to confirm the close relationship of the five species mentioned above, however, in doing so the results indicated that the species *L. ivanovii* could be further subdivided into two sub-species, *ivanovii* and *londoniensis* (Seeliger & Jones, 1988). Table 1.1 shows the two separate lines of descent of the six species of Listeria.

The typical biochemical tests used to distinguish between these 6 species are shown in Table 1.2. The ability of *L. monocytogenes* to produce acid from L-rhamnose but not from D-mannitol, D-xylose or alpha-methyl-D-mannoside can be used to differentiate it from other species of the genus *Listeria*. Other characteristics shown in Table 1.2 include haemolysis, reduction of nitrates to nitrites and the Camp reaction. *Listeria monocytogenes*, *L. ivanovii* and *L. seeligeri* are capable of lysing a wide range of mammalian erythrocytes although in routine haemolysis tests, sheep or horse blood agar plates are used (Skalka *et al.*, 1982; Pine *et al.*, 1987). *Listeria monocytogenes* produces very clear zones of ß-haemolysis when streaked onto blood agar plates, whereas *L. seeligeri* often produce weak or doubtful ß-haemolytic zones. To potentiate any doubtful results obtained from the haemolysis test the Camp reaction is routinely performed (Christie *et al.*, 1944; Rocourt *et al.*, 1983). This test involves streaking cultures of ß-haemolytic *Staphylococcus aureus* or *Rhodococcus equi* vertically onto a sheep blood agar plate and streaking the *Listeria* species being examined at right-angles to these two cultures. The results obtained after 48 hours incubation show enhanced ß-haemolysis by *L. monocytogenes* near the *S. aureus* streak, enhanced ß-haemolysis by *L. ivanovii* near the *R. equi* streak and enhanced haemolysis by *L. seeligeri* near the *S. aureus* streak.

Within a single species further subdivisions can be made based on serological examination. In *L. monocytogenes*, the current knowledge suggests that 16 distinct serological types exist, each varying in the composition of their somatic (O) antigens and their flagellar (H) antigens (Ryser & Marth, 1991). Table 1.3 lists the different serotypes of *L. monocytogenes* in addition to those found in other *Listeria* species. As is evident from Table 1.3, serotypes 1, 3, and 4 were differentiated on the basis of their (O) antigen or H antigen composition (Ryser & Marth, 1991).

Further sub-division of each serotype was based on the discovery of additional O antigens and/or H antigens (Seeliger, 1961; Donker Voet, 1965; Ryser & Marth, 1991). Serologically, *L. innocua, L. ivanovii, L. seeligeri* and *L. welshimeri* are similar to *L. monocytogenes* sharing certain somatic antigens, while *L. grayi* is serologically distinct (Ralovich, 1984). In cases of human listeriosis, 90% of isolates associated with infection belong to serotypes 1/2a, 1/2b and 4b (McLauchlin, 1987; Seeliger & Hohne, 1979).

1.3 PATHOGENESIS

1.3.1 Clinical disease - listeriosis

Of the six currently recognised species in the genus *Listeria*, only two, *L. monocytogenes* and *L. ivanovii* cause the disease known as listeriosis (Hof & Hefner, 1988). *L. monocytogenes* causes the disease in animals and man, although until about 1960 it was associated mainly with infection in animals (Seeliger, 1961; Welshimer, 1981; Donachie & Low, 1995). Human infections by *L. ivanovii* are very rare with only three cases reported to date (Jones *et al.*, 1990). This species is most frequently associated with disease in animals, mainly sheep (Hof & Hefner, 1988), although *L. monocytogenes* is

Table 1.1. Current taxonomic classification of the genus *Listeria*

GENUS	*LISTERIA*	
	FIRST LINE OF DESCENT	SECOND LINE OF DESCENT
SPECIES	*L. monocytogenes*	*L. grayi*
	L. innocua	
	L. ivanovii subspp. *ivanovii*	
	subspp *londoniensis*	
	L. seeligeri	
	L. welshimeri	

Table 1.2. Biochemical Identification of *Listeria* species

Species	Beta-haemolysis	Nitrates reduced	CAMP test SA	CAMP test RE[a]	Production of acid from M	Production of acid from R	Production of acid from X[b]
L. monocytogenes	+	-	+	-	-	+	-
L. ivanovii	+	-	-	+	-	-	+
L. innocua	-	-	-	-	-	v[c]	-
L. welshimeri	-	-	-	-	-	v	+
L. seeligeri	v	-	+	-	-	-	v
L. grayi	-	-	ND	ND	-	-	-

[a]SA = *Staphylococcus aureus*; RE = *Rhodococcus equi*

[b]M = mannitol; R = rhamnose; X = xylose

[c]Variable

Adapted from Ryser & Marth, (1991)

Table 1.3. Serological classification of the genus *Listeria*

SPECIES	SEROTYPE	SOMATIC (O) ANTIGEN TYPE	FLAGELLAR (H) ANTIGEN TYPE
L. monocytogenes	1/2a	I II (III)[a]	AB
	1/2b	I II (III)	ABC
	1/2c	I II (III)	BD
	3a	II (III) IV	AB
	3b	II (III) IV (XII) (XIII)	ABC
	3c	II (III) IV (XII) (XIII)	BD
	4a	(III) (V) VII IX	ABC
	4ab	(III) V VI VII IX X	ABC
	4b	(III) V VI	ABC
	4c	(III) V VII	ABC
	4d	(III) (V) VI VIII	ABC
	4e	(III)V VI (VIII) (IX)	ABC
	7	(III) XII XIII	ABC
L. ivanovii	5	(III) (V) VI (VIII) X	ABC
L. innocua	6a("4f")	(III) V (VI) (VII) (IX) XV	ABC
	6b ("4g")[b]	(III) (V) (VI) (VII) IX X XI	ABC
L. grayi		(III) XII XIV [XVI][c]	E

[a] ( ) = not always present

[b] *L. welshimeri* cannot be differentiated serologically from *L. innocua* serotype 6b

[c] [ ] = proposed by Vazquez-Boland *et al.*, (1992)

Adapted from Ryser & Marth, (1991).

still responsible for approximately 90% of all cases of animal listeriosis (Ryser & Marth, 1991; Donachie & Low, 1995). Not surprisingly, *L. monocytogenes* has attracted greater attention and consequently most investigations are carried out on this species.

The primary means of transmission of *Listeria* to man and animals is through contaminated foodstuffs and this was reported as early as 1915 when an outbreak of a listeriosis-like disease in humans in Australia was linked to food (Atkinson, 1917). Likewise in animals, mainly cattle, outbreaks of listeriosis have been linked to the feeding of contaminated silage (Gray, 1960; Blenden *et al.*, 1966; Fenlon, 1986). In early cases of human listeriosis, no definite foodstuff was established as the source of infection (Seeliger, 1961) although more recent outbreaks have been examined more thoroughly and exact sources have been identified (Gellin & Broome, 1989; Lund, 1990). Foodstuffs such as coleslaw (Schlech *et al.*, 1983), milk (Fleming *et al.*, 1985), various cheeses (Malinverni *et al.*, 1985; Linman *et al.*, 1988), pates (McLauchlin *et al.*, 1991; Goulet *et al.*, 1995), various meats (Anderson & Norrung, 1995; Jacques *et al.*, 1995) and chicken (Gilbert *et al.*, 1989) have been identified as vehicles for transmitting *L. monocytogenes* to humans.

Although contaminated food does seem to be the most important route of transmission, other routes do exist. Infection via the respiratory tract has been reported in both human and animal cases of listeriosis (Odegaard *et al.*, 1952; Wohler & Baugh, 1983) while cutaneous and ocular listeriosis may also occur (Owen *et al.*, 1960; Ralovich, 1984).

The term listeriosis encompasses a wide variety of clinical symptoms which appear to be very similar in animals and man. In animals, outbreaks of listeriosis in herds of domestic animals such as sheep, cattle, goats and swine are of significant worldwide economic importance. The most common form of the disease in adult ruminants appears to be

encephalitis, while in the young, septicaemic infections are more common (Seeliger, 1961; Gitter, 1985; Donachie & Low, 1995). Another form of listeriosis in sheep and cattle is infection of the unborn fetus via the placenta, which usually results in abortion (Gray & Killinger, 1966; Jones & Seeliger, 1991).

In humans, the disease is usually sporadic, although epidemic outbreaks do occur (Gellin & Broome, 1989). Disease is most likely to occur in pregnant women, neonates, elderly and immunocompromised individuals. Infection of healthy adults is rare and may even go unnoticed (Jones & Seeliger, 1991; Farber & Peterkin, 1991). The clinical symptoms may range from mild influenza-like illnesses to meningitis and meningoencephalitis. The most commonly reported symptoms are meningeal related, although in pregnant women the most likely symptom to occur is a mild influenza-like illness.

The exact incidence of listeriosis worldwide is not known, mostly due to the fact that the clinical symptoms of mild listeriosis are non-specific thereby allowing cases to go undetected. However, estimates of the number of cases to range from 1 - 12 incidences per million of population (Gellin & Broome, 1989; Jones, 1990) although these figures are most definitely on the increase (Broome *et al.*, 1990; Campbell, 1990). This increase in cases of listeriosis in recent years has led to renewed interest in the *Listeria*, in particular the pathogenicity of these organisms.

1.3.2 Immunity and treatment against *L. monocytogenes*

The immune response to *L. monocytogenes* includes both humoral and cell mediated responses. A strong antibody response to a number of *L. monocytogenes* surface proteins and listeriolysin O is stimulated, however, it has been shown that these antibodies alone do not protect against infection (Njoki-Obi & Osebold, 1962; Beattie *et al.*, 1990; Barry

et al., 1992). The ability of antibodies to opsonise *Listeria* cells and stimulate the classical complement pathway has been implicated as an important immune response to infection, however, in neonates, there is a much lower activity of this pathway due to lesser amounts of IgM opsonic antibody and this may contribute to the increased susceptibility of this group to infection by *L. monocytogenes* (Issekutz *et al.*, 1984; Bortolussi *et al.*, 1986).

Although the level of antibody production does appear to affect the susceptibility of certain individuals such as pregnant women and neonates to *Listeria* infection, the most important immune response to *L. monocytogenes* is the cell mediated response. Recovery from listerial infection is associated with the development of acquired cellular resistance (Mackaness, 1969; Hahn & Kaufmann, 1981; Kaufmann, 1984) which is supported by the fact that transfer of resistance to *L. monocytogenes* can be obtained via lymphoid cells and also that immunodeficient individuals have a greater likelihood of contracting listeriosis (Mackaness, 1969; Gellin & Broome, 1989).

Early research into immunisation against listeriosis in animals involved mainly bacterin, as sheep vaccines, however, the results indicated that no protection was obtained even when large doses of the bacterin were administered (Graham *et al.*, 1943). In later investigations, killed vaccines were examined but the results were rather contradictory. Many workers failed to report induction of a protective immune response (Kearns & Hinrichs, 1977; Wirsing von Koenig *et al.*, 1982; Berche *et al.*, 1987), although one group reported that by using extremely large doses of non-viable *L. innocua*, protective immunity was induced in mice (Wirsing von Koenig *et al.*, 1983).

The fact that replicating cells are more efficient in stimulating an immune response that non-replicating cells (Hahn & Kaufmann, 1981) and that intracellular growth was

reported as a pre-requisite for induction of cell-mediated protection (Baldrige *et al.*, 1988; Brunt *et al.*, 1990) led to the use of live vaccines. More success was achieved when attenuated live vaccine strains were used for immunisation (Kearns & Hinrichs 1977; Ivanov, 1981; Linde *et al.*, 1991). Early studies (Ivanov *et al.*, 1977) employed bacteriostatic agents in order to attenuate *Listeria* strains, however, more recent investigations have used strains with naturally reduced virulence, as this avoided the possible problems of antigenic structure alteration which may occur when using bacteriostatic agents (Michel *et al.*, 1990). Listeriolysin O-negative *Listeria* strains have been suggested as possible vaccine strains (Michel *et al.*, 1990), however, the fact that they lack LLO presented problems as this factor is a major stimulant of the immune response and protection against *L. monocytogenes* (Berche *et al.*, 1987; Barry *et al.*, 1992). Thus, the current approach in identifying an ideal vaccine is the use of auxotrophic mutants (Alexander, 1994) similar to those employed with a number of Gram negative bacteria (Dougan *et al.*, 1987; O'Callaghan *et al.*, 1988; Mukkur *et al.*, 1991).

1.3.3 Virulence determinants

Despite significant advances in recent years, the current knowledge of the pathogenesis of listeriosis remains incomplete. Like most pathogenic organisms the mechanisms by which *L. monocytogenes* causes disease is highly complex, involving many different stages. The pathogenesis of listeriosis can be broadly divided into three main steps - 1. penetration of the host, 2. survival and multiplication of the organism within the host and 3. invasion of target tissue (Ryser & Marth, 1991; Sheenen *et al.*, 1994; Dramsi *et al.*, 1996). Due to the characteristic penetration of the intestine by *L. monocytogenes*, these organisms have been classified as enteroinvasive pathogens (Mackaness, 1969; Racz *et al.*, 1970). During the final stages of this disease, excape from host macrophages a

septicaemia may occur allowing the organism to spread to body organs, such as the brain, (Jones & Seeliger, 1991; Ryser & Marth, 1991).

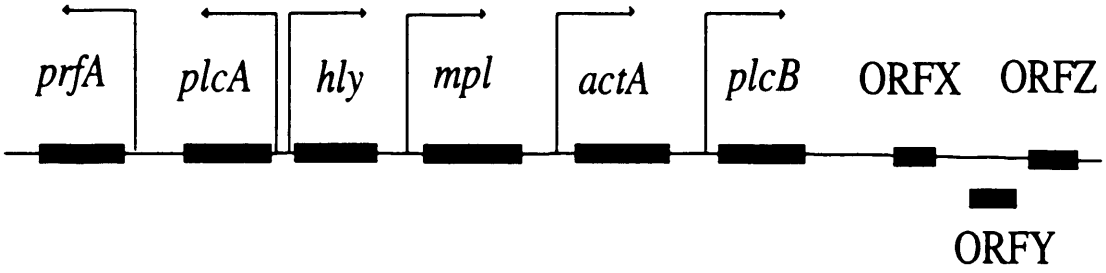
In order to penetrate, survive and multiply within the host and eventually cause disease, most pathogens possess a number of different virulence determinants which are involved at the different stages of the disease (Finlay & Falkow, 1989). The application of molecular biological techniques, in particular gene cloning and transposon mutagenesis, has allowed identification and characterisation of such determinants and has led to a better understanding of their role in the pathogenesis of disease (Macrina, 1984). Virulence determinants, both cell-associated and extracellular, have been described in *L. monocytogenes* and include: listeriolysin O, phospholipases, catalase, superoxide dismutase internalin, monocyte producing activity (MPA), immunosuppresive activity (ISA), delayed-type hypersensitivity factor (DTH) and protein p60 (Chakraborty & Goebel, 1988; Goebel *et al.*, 1991, Portnoy *et al.*, 1992; Sheenen *et al.*, 1994; Dramsi *et al.*, 1996). The encoding genes for some of these determinants have been identified, cloned and several are located on an operon lying adjacent to the listeriolysin encoding gene, *hly* (Portnoy *et al.*, 1992). The arrangement of these genes is shown in Figure 1.1

1.3.3.1 Extracellular factors

1.3.3.1.1 Listeriolysin O - LLO (*hly*)

The *L. monocytogenes* haemolysin, listeriolysin O, is the most characterised virulence determinant of the species. All pathogenic strains of this organism are haemolytic (Liu & Bates, 1961). Although there is strain variation in the level of haemolytic activity, this is not correlated with virulence (Kathariou *et al.*, 1988). Listeriolysin O is a member of a family of thiol-activated pore-forming cytolysins of which streptolysin O, from

Figure 1.1. The *Listeria monocytogenes* listeriolysin and lecithinase operon. Diagrammatic representation of the gene arrangement of a number of listerial virulence determinants. Most of the genes are discussed in this section of the thesis.



prfA: positive regulator

plcA: phosphatidylinositol-specific phopholipase C

hly : listeriolysin O

mpl : mettaloprotease

actA: surface protein involved in actin assembly

plcB: lecithinase

ORFX: unknown function

ORFY: unknown function

ORBZ: unknown function

(Adapted from Portnoy *et al.*, 1988; Sheenen *et al.*, 1994)

Streptococcus pyogenes is the prototype (Njoki-Obi *et al.*, 1963; Parrisuis *et al.*, 1986; Gaillard *et al.*, 1987). This protein, is a heat-labile single polypeptide of approximate molecular weight 60 kDa and is encoded by the *hly* gene (Njoki-Obi, 1963; Cossart, 1988; Goebel *et al.*, 1988).

The essential role of LLO for virulence was shown by means of gene cloning and transposon mutagenesis experiments. Non-haemolytic mutants of *L. monocytogenes* were shown to be completely avirulent (Gaillard *et al.*, 1986; Kathariou *et al.*, 1987; Kuhn *et al.*, 1990) while introduction of the cloned gene on a plasmid into a strain containing a structural gene mutation, resulted in restoration of virulence (Cossart *et al.*, 1989; Mengaud *et al.*, 1991). Electron microscopy studies of non-haemolytic mutants have shown that the mutants reside in the endosomal vacuole of Caco-2 cells, do not escape into the cytoplasm and consequently are unable to multiply intracellularly (Gaillard *et al.*, 1987) in contrast to the wild-type bacterium. Additional evidence supporting the role of LLO in the lysis of the phagosomal membrane comes from experiments involving the cloning of the listerial *hly* into a non-invasive *Bacillus subtilus* and also into an attenuated *Salmonella dublin* (Bielecki *et al.*, 1990; Gentschev *et al.*, 1995). Both these altered organisms were capable of invading macrophages, lysing phagosomal membranes and growing intracellularly. Other workers, however, suggested that factors other than LLO may be involved in escape from the phagolysosome as they found that non-haemolytic mutants could grow in human epithelial cells, (Portnoy *et al.*, 1988; Jones & Portnoy, 1994; Goldfine *et al.*, 1995; Marquis *et al.*, 1995).

Following penetration of the host, *L. monocytogenes* is phagocytosed into an iron-poor environment (Bullen, 1981) which has been shown to stimulate production of LLO (Cowart & Foster, 1985; Cowart, 1987). The acidic pH conditions presumed to exist within the phagolysosome have also been shown to be able to stimulate listeriolysin

activity (Geoffroy *et al.*, 1991). Expression of *hly* is known to be positively regulated by the virulence regulator, *prf*A although prfA-independent regulation of expression of hly has been reported (Portnoy *et al.*, 1992; Sheenan *et al.*, 1994; Bohne *et al.*, 1994) . Thus, *in vivo*, it is thought that LLO, and maybe other factors, mediate intracellular lysis of the phagosome and release of the bacterium into the cytoplasm which is a more favourable environment for bacterial growth (Gaillard *et al.*, 1987; Kuhn *et al.*, 1990)

1.3.3.1.2 Phosphatidylinositol-specific phospholipase C - PI-PLC (*plcA*)

Adjacent to *hly* is a gene *plcA* which encodes phophatidylinositol-specific phopholipase C, PI-PLC (Leimeister-Wachter *et al.*, 1991; Goldfine & Knob, 1992). This virulence factor was identified during screening of *L. monocytogenes* haemolysin-negative mutants, where some of the mutants were able to escape the intracellular vacuole and form plaques in cell lines in tissue culture (Kathariou *et al.*, 1990). This property was associated with the ability to produce PI-PLC (Mournier *et al.*, 1990), whose gene, *plcA*, encodes a protein of approximate molecular weight 36 kDa (Camilli *et al*, 1991; Leimeister - Wachter *et al.*, 1991; Mengaud *et al.*, 1991). The protein shows approximately 30 % amino acid identity to the PI-PLC of *Bacillus thuringiensis* and *Bacillus cereus* (Low, 1990) and like these other enzymes it has been suggested that PI-PLC hydrolyses PI and glycosyl-PI anchored proteins of the phagolysosomal membrane facilitating lysis of the membrane by LLO (Goldfine & Knob, 1992). Interestingly, these proteins are only produced by the pathogenic species of the genus *Listeria*, namely, *L. monocytogenes* and *L. ivanovii* (Notermans *et al.*, 1991).

The exact role of this enzyme *in vivo* was investigated (Camilli *et al.*, 1991; Mengaud *et al.*, 1991) using *plc*A insertional mutants which were shown to have reduced virulence in mice, however, these latter workers proposed that the reduction in virulence may actually

be due to a polar affect of the mutation on the downstream regulatory element *prfA* (Mengaud *et al.*, 1991). More recent studies (Camilli *et al.*, 1993; Smith *et al.*, 1995; Marquis *et al.*, 1995) have involved in-frame deletion mutations in order to minimise the polar effect of the mutation on downstream genes. They found a clear correlation of the absence of PI-PLC with a decreased ability to lyse host vacoules.

The overall implications from these various reports are that PI-PLC is an important virulence determinant of *L. monocytogenes*, and acts in combination with LLO to lyse vacoular membranes, although the importance of each may vary depending on the cell type invaded (Marquis *et al.*, 1995; Goldfine *et al.*, 1995).

1.3.3.1.3 Metalloprotease (*mpl*)

The gene which encodes the metalloprotease is designated *mpl* and lies immediately downstream from the *hly* gene and was shown to be found only in pathogenic strains of *L. monocytogenes* (Domann *et al.*, 1991). The *mpl* gene product has been detected in culture supernates as a 60 kDa protein, which represents the immature, inactive form of the protein while the mature form could only be isolated from one strain, NCTC 7973 (Domann *et al.*, 1991). The metalloprotease contains significant amino acid homology to a family of metalloproteases of which thermolysin from certain *Bacillus* species is the prototype (Domann *et al*, 1991; Mengaud *et al*, 1991). Insertional mutations in *mpl* have been shown to exert a polar effect on *actA* and *plcB* (Mengaud *et al.*, 1991; Raveneau *et al.*, 1992; Poyart *et al.*, 1993) and consequently have hampered attempts to assign an exact role of this gene product in listerial virulence. Some reports, however, have suggested that *mpl* mutants have reduced virulence and are only capable of producing a 33 kDa form of the lecithinase protein (see below), implying that the metalloprotease

may be involved in proteolytic processing of lecithinase (Poyart *et al.*, 1993; Marquis *et al.*, 1995).

1.3.3.1.4 Lecithinase (*plcB*)

The lecithinase encoding gene, *plcB*, predicts a protein of 289 amino acids with sequence similarity to phosphatidylcholine (PC)-PLC of *B. cereus* and *Clostridium perfringens* (Vazques-Boland *et al.*, 1992). Listerial lecithinase is a broad spectrum PLC with the ability to catalyse hydrolysis of PC, phosphatidylethanolamine (PE), glycosyl-PI, to a lesser extent sphingomyelin and very weakly PI (Geoffroy *et al.*, 1991; Goldfine *et al.*, 1993). Two forms of the enzyme have been described: an inactive form equivalent to a 33 kDa protein and an active form represented by a 29 kDa protein (Neibuhn *et al.*, 1993). The 29 kDa protein is thought to be produced by cleavage of the 33 kDa protein by metalloprotease (Poyart *et al.*, 1993). The ability to produce lecithinase is associated with virulence and pathogenic listerial strains and is influenced by various environmental parameters including temperature, salt and pH (Sheenan *et al.*, 1994).

Transposon mutagenesis studies have provided evidence to support the role of lecithinase in the breakdown of the double membrane which surrounds the bacteria during cell-to-cell spread (Vazques-Boland *et al.*, 1992). Transposon mutants of *plcB* were shown to produce smaller plaques than the wild type on fibroblast monolayers. However, the fact that the mutant still produced plaques suggested that other factors are involved, namely LLO and PI-PLC (Marquis *et al.*, 1995).

1.3.3.1.5 Protein p60 (*iap*)

The *iap* gene of *L. monocytogenes* encodes a major extracellular protein of 60 kDa, p60 (Kuhn & Goebel, 1989; Kohler *et al.*, 1990). Studies of mutants which produced reduced levels of p60 and also formed cell filaments showed them to have impaired invasiveness of cell lines in tissue culture (Sun *et al.*, 1990; Goebel *et al.*, 1991). Bacteria which form these filaments have been referred to as rough (R) mutants (Kuhn & Goebel 1989; Kohler *et al.*, 1990) and have been found to occur spontaneously. Recent evidence suggested a role for this protein in the adherence and invasion of mouse fibroblasts by *L. monocytogenes* (Bubert *et al.*, 1992; Wuenscher *et al.*, 1993). Expression of p60 in *B. subtilus* DB104 and *Salmonella typhi* (Wuenscher *et al.*, 1993; Hess *et al.*, 1995), both of which have the same characteristics as rough *L. monocytogenes* mutants, led to a disruption of the cell chains normally produced by these strains and also a promotion of invasion of various cell types by these otherwise non-invasive rough mutants. Thus, p60 can be considered an important listerial virulence determinant, involved in invasion of host cells, although it may also be an important housekeeping gene as complete inactivation of this gene causes the cells to lose viability (Wuenscher *et al.*, 1993).

1.3.3.1.6 Catalase and superoxide dismutase (SOD)

It has been postulated that catalase and SOD of *L. monocytogenes* may be regarded as virulence factors because of their ability to inactivate reactive molecules, O_2^- and H_2O_2, produced during the hosts immune response. The genes encoding a catalase from *L. seeligeri* and SODs from *L. monocytogenes* and *L. ivanovii* have been cloned (Haas *et al.*, 1991; Brehm *et al.*, 1992). The SOD encoding genes predicts a 202 amino acid protein with strong homology to SODs of other bacteria including, *Staphylococcus aureus* and *Streptococcus pneumoniae* (Poyart *et al.*, 1995). The gene is also conserved among other

Listeria species, although variation in the overall amount of protein produced by the different species does occur (Vasconcelos& Deneer, 1994). The importance of catalase in the pathogenicity of *L. monocytogenes* was investigated using transposon mutagenesis and the results suggested that catalase-negative mutants were less virulent (Leblond-Francillard *et al.*, 1989). However, further experiments are certainly required to confirm the role of both these enzymes in the pathogenesis of listeriosis.

1.3.3.2 Cell surface components

1.3.3.2.1 Internalin (*inlA*)

Invasion of host epithelial cells is a major step in the pathogenesis of listeriosis and a factor responsible for this invasion has been identified (Gaillard *et al.*, 1991). Transposon insertion upstream of a genomic locus named *inl* resulted in *L. monocytogenes* being unable to invade cultured epithelial cells and also, introduction of the gene *inlA* into *L. innocua* enabled this non-invasive species to invade epithelial cells (Portnoy *et al.*, 1988). The *inlA* gene encodes internalin, an 800 amino acid protein which belongs to a family of surface proteins of which *S. pyogenes* M protein is a member (Fischetti *et al.*, 1990). Characteristic features such as a signal sequence, two different repeat regions, a carboxy-terminal hydrophobic region and a hexapeptide CPTTGD, together with Western blot analysis and immunogold labelling, suggested that internalin is a cell wall associated protein (Gaillard *et al.*, 1991; Dramsi *et al.*, 1993).

The other gene of the *inl* locus, *inlB*, lies downstream and is cotranscribed with *inlA*. It encodes a 630 amino acid protein with very similar features to the *inlA* protein except for the carboxy terminal region which serves to guide the *inlA* gene product to the cell wall (Navarre & Schneewind, 1994). The role of internalin *in vivo* was assessed using

isogenic mutants and the result showed that both *inlA* and *inlB* mutants had reduced virulence compared with the wild type (Dramsi *et al.*, 1994). Recently, other genes with homology to *inlA* have been identified in *L. monocytogenes* and it has been suggested that this family of internalin genes encodes surface proteins with different specificities for different cell types (Dramsi *et al.*, 1994).

1.3.3.2.2 Monocytosis producing agent (MPA)

The importance of MPA in listerial pathogenicity is not fully understood although the characteristic monocytosis which occurs in animal listeriosis was shown to be induced by this factor. However, in humans MPA does not induce monocytosis although it is known to damage macrophages (Shum & Galsworthy, 1982). Earlier work reported that the MPA is in fact a lipid of molecular weight 1.0 kDa which is found associated to the plasma membrane (Galsworthy et al., 1977) but to understand its role in virulence further studies have to be performed.

1.3.3.2.3 Delayed-type hypersensitivity (DTH) reaction (*lmaBA* operon)

The *lmaA* gene was shown to encode a 20 kDa cell wall-associated protein which was capable of inducing a specific delayed-type hypersensitivity reaction (Gohmann *et al.*, 1990). Pathogenic and non-pathogenic strains of *Listeria* are able to induce this DTH reaction although the *lmaA* gene is found uniquely in pathogenic species, *L. monocytogenes* and *L. ivanovii* (Hof & Chatzipanagiotou, 1987). This suggested that other factors, in addition to *lmaA* gene product, are likely to be involved in eliciting this reaction. The *lmaB* gene encodes a 14 kDa polypeptide although the precise role of the *lmaBA* operon remains to be determined (Portnoy *et al.*, 1992).

1.3.3.2.4 Immunosuppressive agent (ISA)

ISA is a cell surface component of *L. monocytogenes* and was recognised due to the fact that spleen cells from *Listeria*-infected animals were unable to synthesise antibody (Kim *et al.*, 1976). ISA is composed of amino acids, carbohydrate, phosphorous, glycerol and has a molecular weight of 150 kDa and has been reported to act, *in vivo*, as a B-cell mitogen, stimulating the production of suppressor T-cells (Galsworthy, 1987). A full understanding of its role in listerial pathogenicity awaits further investigations.

1.3.3.2.5 *actA*

The final virulence determinant to be discussed here is that which is encoded by the *actA* gene, the second gene of the lecithinase operon - see Figure 1.1 (Vazques-Boland *et al.*, 1992). The sequence of *actA* predicts a protein of approximately 610 amino acids, with a molecular weight of 90 kDa, which is found at the surface of the cell (Domann *et al.*, 1991). Transformation of an *actA* -negative mutant with *actA* on a plasmid has shown that the gene product is involved in actin assembly. Also, *actA* mutants do not nucleate the polymerisation of actin filaments (Kocks *et al.*, 1995). Whether or not the protein is actually an actin nucleator or has another function is not known.

Having discussed the various virulence factors produced by *L. monocytogenes*, it is evident that the pathogenesis of this disease is multifactorial. This multifactorial nature of disease is common throughout bacterial pathogens, where many factors are involved at the different stages, some of these being more important and more fully understood than others. The expression of these determinants at different stages in the pathogenesis of disease has been shown to be highly influenced by environmental signals including temperature, osmolarity, anaerobiosis, pH and iron (Maurelli, 1989; Griffiths, 1991;

Mekalanos, 1992) suggesting that bacterial pathogens, including *L. monocytogenes*, must be able to sense and adapt to the surrounding environments encountered at the different stages in the pathogenesis of disease. In addition, they must also be able to acquire nutrients essential for survival and proliferation in these environments.

1.4 IRON - ITS IMPORTANCE AND ACQUISITION

1.4.1 Iron and infection

One important nutrient which almost all bacteria have been shown to require for survival and proliferation is iron (Otto *et al.*, 1992; Williams & Griffiths, 1992; Griffiths, 1993). Non-pathogenic lactobacilli are the only microorganisms which have been shown to survive in the absence of iron (Archibald, 1983). This metal is known to be an important component of many bacterial enzymes including, ribonucleotide reductase, RNA polymerase III, nitrogenase, cytochromes (Williams, 1990; Wooldridge & Williams, 1993), all of which are involved in cellular functions. The importance of iron is due to the extremely wide redox potential conferred by the two forms of this ion (Neilands, 1981): ferric iron (Fe^{3+}) and ferrous iron (Fe^{2+}). In the presence of O_2, iron is oxidised to the ferric state and may form insoluble ferric hydroxides, both forms of which are insoluble. In mammalian hosts, iron is maintained in a soluble form by being bound to carrier molecules or being reduced to ferrous iron (Weinberg, 1984).

Most animal and human hosts have developed iron-withholding mechanisms which are able to provide essential iron to host cells but prevent invading microbes receiving the iron (Weinberg, 1995). These iron-withholding mechanisms can be divided into two main groups: (1) those which are constitutively expressed and (2) those which are induced by microbial infection (Weinberg & Weinberg, 1995).

Constitutively expressed iron-withholding components produced by mammalian hosts include haemoglobin, haem, ferritin, haemosiderin which bind intracellular iron and transferrin and lactoferrin which are responsible for binding extracellular iron in serum and body fluids (Otto *et al.*, 1992; Litwin & Calderwood, 1993). In mammals, most iron is found intracellularly, complexed with haemoglobin (Otto *et al.*, 1992), although following lysis of erythrocytes, haemoglobin and haem are released and have been found to serve as iron sources for many bacteria. However, the released haemoglobin and haem are rapidly bound by serum proteins, haptoglobin and haemopexin, respectively, and removed from the circulation (Litwin & Calderwood, 1993). The amount of free iron which occurs in normal human serum was reported as being 10^{-18} M (Bullen *et al.*, 1978), a level which is bacteriostatic and presents problems for invading bacteria. It is interesting that during microbial infection, humans reduce this already low level of iron even further, in a series of reactions known as the hypoferraemic response (Weinberg, 1978; Weinberg, 1984). These reactions are thought to be mediated by interleukin-1 (IL-1) and tumour necrosis factor alpha (TNF-α) (Dinarello, 1984; Beutler & Cerami, 1987) and result in increased production of transferrin, lactoferrin and ferritin, with a consequent reduction in free iron, both intra- and extracellularly (Weinberg, 1984).

The importance of this further reduction in available iron, in terms of resisting infection, has been questioned (Brock & Mainou-Fowler, 1986). It is thought that lowering the level of iron in the intracellular environments during the hypoferraemic response is of greater significance than the reduction which occurs in the extracellular environment because extracellular pathogens have been shown to use iron from intracellular environments which are considered iron-rich (Brock *et al.*, 1991).

The importance of iron in infection has also been demonstrated in numerous studies which involved injection of exogenous iron into animals to produce iron-overload. It was

suggested that as a consequence of this iron-overload, these animals showed increased susceptibility to bacterial infection (Sword *et al.*, 1966; Bullen *et al.*, 1978; Field *et al.*, 1986). Likewise, humans who have suffered from haemochromatosis, chronic transfusions, or oral iron overdose, all of which resulted in increased iron levels, become more susceptible to bacterial infections (Blei & Pruder, 1993; Monno *et al.*, 1994; Vadilo *et al.*, 1994; Kessler *et al.*, 1993). Thus, the *in vivo* environments of most healthy mammals have restricted availability of iron which invading bacterial pathogens must adapt to, and acquire iron from, if they are to survive and multiply.

1.4.2 Bacterial Iron Acquisition (excluding Listeria)

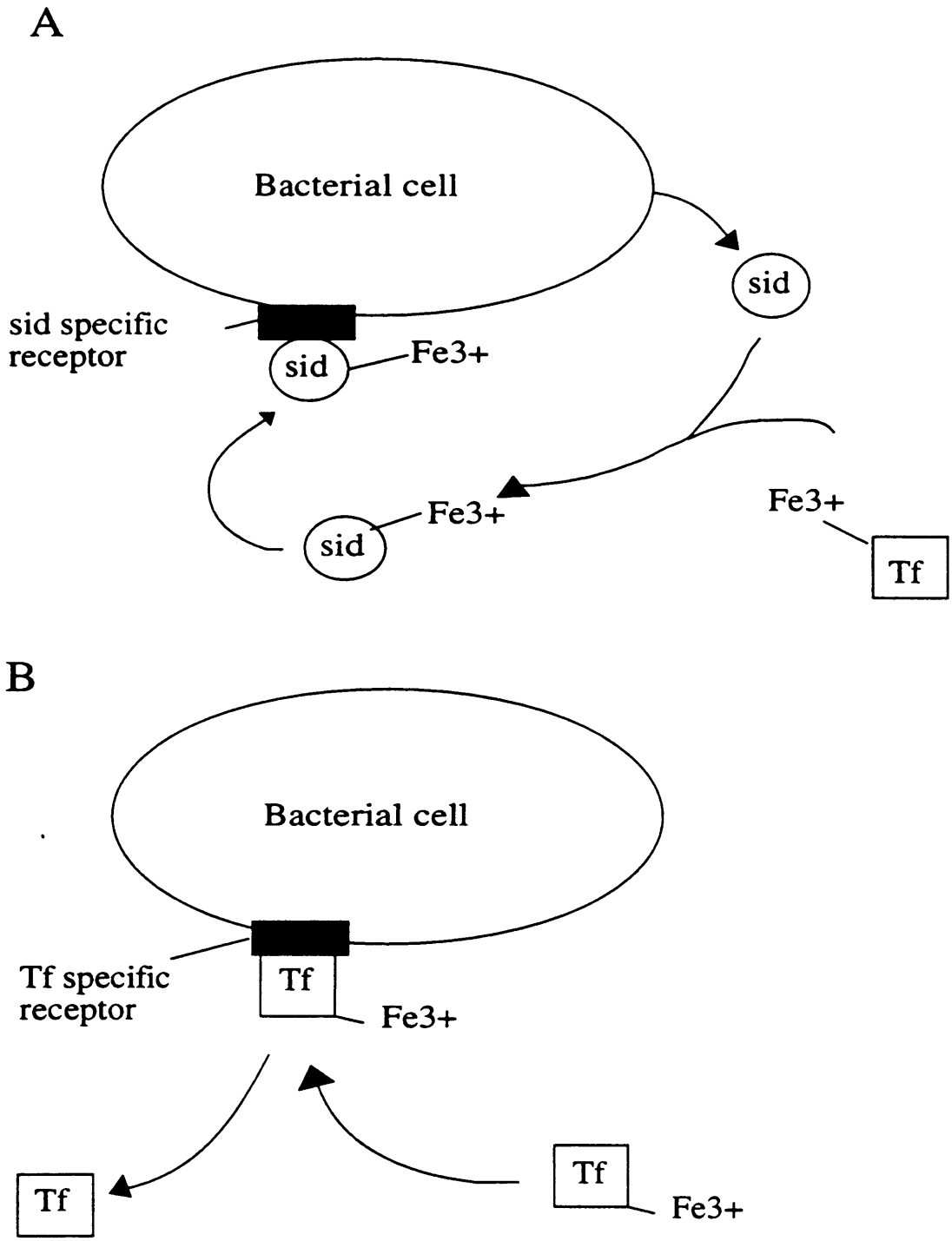
A number of mechanisms have been identified whereby bacteria sequester iron from host iron-binding proteins and they can be broadly divided into two main groups: (1) siderophore-dependent and (2) siderophore-independent mechanisms, as shown in Figure 1.2 (Griffiths *et al.*, 1992).

The best understood mechanism involves the production of iron chelators, known as siderophores. These molecules, produced by Gram positive and Gram negative bacteria (Griffiths *et al.*, 1992; Otto *et al.*, 1992) are low-molecular weight proteins with a high affinity for ferric iron and are able to sequester ferric iron from host transferrin and lactoferrin making it available for use by the organism.

1.4.2.1 Siderophore-dependent iron acquisition

Siderophores are secreted by bacteria in response to iron deprivation and once complexed to ferric iron which has been acquired from host iron binding proteins they are

Figure 1.2. Bacterial iron uptake mechanisms. Simplified diagrams of siderophore-dependent (A) and siderophore-independent (B) iron acquisition are shown. "sid" represents the siderophore molecules while "Tf" represents transferrin. Transferrin is used in these diagrams to represent a ferric iron chelator which could also be represented by lactoferrin, haem or other iron chelators. These diagrams are described in more detail in the text.



(Adapted from Griffiths et al., 1987)

transported back into the cell via specific outer membrane receptors which are themselves also regulated by iron (Figure 1.2A). There are currently two chemically distinct types of siderophores: (1) phenolates and (2) hydroxamates, examples of which are enterochelin and aerobactin, respectively (Rogers *et al.*, 1977; Payne, 1988). These are produced by various members of the family *Enterobacteriaceae*. In *E. coli*, the genes responsible for the biosynthesis, transport, and hydrolysis of enterochelin and aerobactin are found in gene clusters which are regulated in response to surrounding iron levels by the *fur* gene product (Neilands, 1981; Crosa, 1984). Similar systems have been identified in other enteric bacteria including, *Aerobacter aerogenes* (Gibson & Magrath, 1969), *Klebsiella pneumoniae* (Nassif & Sansonetti, 1986) and *Shigella flexneri* (Griffiths *et al.*, 1985). More recently, enterics such as Proteus, Providencia and Morganella, have been shown to produce a-keto- and a-hydroxy acids which act in a similar manner to siderophores of other bacteria (Dreschel *et al.*, 1993), sequestering iron for bacterial usage.

Siderophore-dependent iron acquisition in non-enteric bacteria has been identified and has been most widely studied in *Vibrio*, *Pseudomonas*, Mycobacterial and *Staphylococcal* species. *Vibrio anguilarrium* has been shown to produce a phenolate siderophore, anguibactin (Crosa, 1984) while *Vibrio cholerae* strains produce vibriobactin, also a phenolate siderophore (Griffiths *et al.*, 1984). Pyoverdin and pyochelin are produced by *P. aeruginosa* (Cox & Adams, 1985) while *Staphylococcal* species produce staphyloferrins (Konetschny-Rapp *et al.*, 1990) and Mycobacterial species produce mycobactin and exochelin (Stritharam & Ratledge, 1988). Cell receptors for these siderophores have been identified (Sokol & Woods, 1983; Hall *et al.*, 1987) and like enteric siderophore receptors, their expression was shown to be repressible by iron (Weinberg, 1989). Interestingly, it has been reported that some microorganisms are capable of obtaining iron from siderophores which are not produced by themselves but by other so-called "foreign" sources (Baig *et al.*, 1986; Field *et al.*, 1986; Williams et al.,

1990). A summary of the siderophores produced by various bacteria is shown in Table 1.4.

The importance of the two different types of siderophores for bacterial growth and virulence has been investigated in *E. coli* (Brock *et al.*, 1991). *In vitro* studies suggested that enterochelin was a more effective siderophore than aerobactin as the stability constant of ferric-enterochelin was significantly higher than that of ferric-aerobactin (Bagg & Neilands, 1987). However, other investigators suggested that aerobactin was a more efficient siderophore as it could stimulate bacterial growth at a concentration 500 times less than enterochelin (Braun *et al.*, 1984). Brock *et al.*, (1991) suggested that the production of both siderophores would prove advantageous to the bacterium as they both have affinities for different iron sources. *In vivo*, siderophores have been shown to be important for virulence of a number of bacteria including, *E. coli* (Montgomerie *et al.*, 1984), *V. cholerae* (Sigel *et al.*, 1985), and *P. aeruginosa* (Cox, 1982). This association of virulence with siderophore production was made due to the non-virulent characteristics of siderophore mutants of different bacteria (Sigel *et al.*, 1985) and to the increased prevalence of siderophores in disease-associated bacterial strains (Carbonetti *et al.*, 1986; Jacobson *et al.*, 1988)

1.4.2.1 Siderophore-independent iron acquisition

Not all pathogens are capable of producing siderophores, therefore it is not surprising that siderophore-independent mechanisms have been identified. The best understood of these and those which have attracted most research are those which involve expression of membrane proteins, under iron restriction, which are specific for host transferrin or lactoferrin molecules - see Figure 1.2B (Williams & Griffiths, 1992). This mechanism has been identified in species belonging to the family *Pasteurellaceae* (Morton &

Table 1.4. Mechanisms of iron uptake by bacterial pathogens

Species	Mechanism		Reference
	Siderophore-dependent	Siderophore-independent	
Escherichia coli	enterochelin, aerobactin	ferric citrate, haem	Payne *et al.*, (1988)
Klebsiella pneumoniae	enterochelin aerobactin		Nassif & Samson, (1986)
Salmonella spp.	enterochelin aerobactin a-keto/hydroxy-acid		Pollock & Neilands, (1970) Kingsley *et al.*, (1996)
Serratia spp.	aerobactin		
Shigella spp.	enterochelin aerobactin		Martinez *et al.* (1987)
Mycobacterium smegmatis	exochelin mycobactin		Stritharam & Ratledge, (1988)
Mycobacterium leprae	exochelin		Hall *et al.*, (1987)
Vibrio cholerae	vibriobactin	haem, haemoglobin	Stoebner & Payne, (1988)
Pseudomonas aeruginosa	pyoverdin, pyochelin enterochelin		Cox & Adam, (1985)
Staphylococcus hyicus	staphyloferrins A, B a-ketoacids		Marcelis *et al.*, (1978) Heuck *et al.*, (1995)

Table 1.4 continued. Mechanisms of iron uptake by bacterial pathogens

Species	Mechanism: Siderophore-dependent	Mechanism: Siderophore-independent	Reference
Neiserria meningitidis		haem, haemoglobin TBP, LBP	Genca & Desai, (1996)
Neiserria gonococci	aerobactin	haem, haemoglobin TBP, LBP	Genca & Desai, (1996)
Haemophilus influenzae	enterochelin	haem, haemoglobin TBP	Schryvers, (1989)
Actinobacillus pleuropneumoniae		TBP	Ogunnariwo *et al.*, (1991)
Aeromonas salmonicida		TBP, LBP	Chart & Trust (1983)
Bordetella pertussis		TBP, LBP	Redhead *et al.*, (1987)
Legionella pneumophila		reductant	Johnson *et al.*, (1991)
Streptococcus mutans		reductant	Evans *et al.*, (1986)
Bacillus subtilus		reductant	Gaines *et al.*, (1981)

Williams, 1989; Ogunnariwo *et al.*, 1991; Ricard *et al.*, 1991) and also in *Neisseria* species, meningococci (Archibald & DeVoe, 1979) and gonococci (Norrod & Williams, 1978).

Transferrin and lactoferrin receptors of *N. meningitidis* and *N. gonococci* have been shown to be specific for human transferrins and lactoferrins (Schryvers & Morris, 1988; Schryvers & Lee, 1989). Two transferrin binding proteins (TBPs) of 98 and 70 kDa (Schryvers & Lee, 1989) and one lactoferrin binding protein (LBP) of 105 kDa (Schryvers & Morris, 1988) have been isolated from *N. meningitidis*. The larger TBP is highly conserved among different isolates while the smaller one varies both in antigenicity and size (Genco & Desai, 1996). Similarly, in *Haemophilus influenzae* and other *Haemophilus* species, two TBPs have been identified with sizes and properties similar to those of *N. meningitidis* (Schryvers, 1989; Ogunnariwo *et al.*, 1991; Genco & Desai, 1996).

In other bacterial species, including *Actinobacillus pleuropneumoniae* (Gonzales *et al.*, 1990), *Bordetella pertussis* (Redhead *et al.*, 1987), *Aeromonas salmonicida* (Chart & Trust, 1983) the ability to utilise transferrin or lactoferrin-bound iron has also been documented. A list of TBP and/or LBP-producing organisms is shown in Table 1.4.

Other mechanisms of iron acquisition include (1) the ability to use iron bound to haem or haemoglobin found in serum following lysis of erythrocytes (Perry & Brubaker, 1979; Pidock *et al.*, 1988), (2) the utilisation of iron bound to citrate (Payne, 1988), and (3) the production of a ferric reductase capable which results in the release of the reduced ferric iron from host iron binding proteins (Gaines *et al.*, 1981; Evans *et al.*, 1986; Johnson *et al.*, 1991) - see Table 1.4.

1.4.3 Iron acquisition in *L. monocytogenes*

One of the earliest studies on the importance of iron for the growth of *L. monocytogenes* involved both *in vitro* and *in vivo* analysis of listerial growth under different iron concentrations (Sword, 1966). A stimulatory effect on growth, proportional to iron concentration, be it ferric iron (ferric ammonium citrate) or ferrous iron (ferrous sulphate), was reported following *in vitro* analysis (Sword, 1966). *In vivo*, pre-injection of mice with either ferric or ferrous iron, significantly reduced the LD_{50} of *L. monocytogenes* and not surprisingly increased the bacterial numbers isolated from liver and spleen tissues of these animals (Sword, 1966). Thus from this early period, the importance of iron in relation to listerial growth and infection was apparent, however, until the last decade no further investigations were performed. The studies which have now been documented have led to a clearer but still incomplete understanding of iron uptake by *L. monocytogenes*.

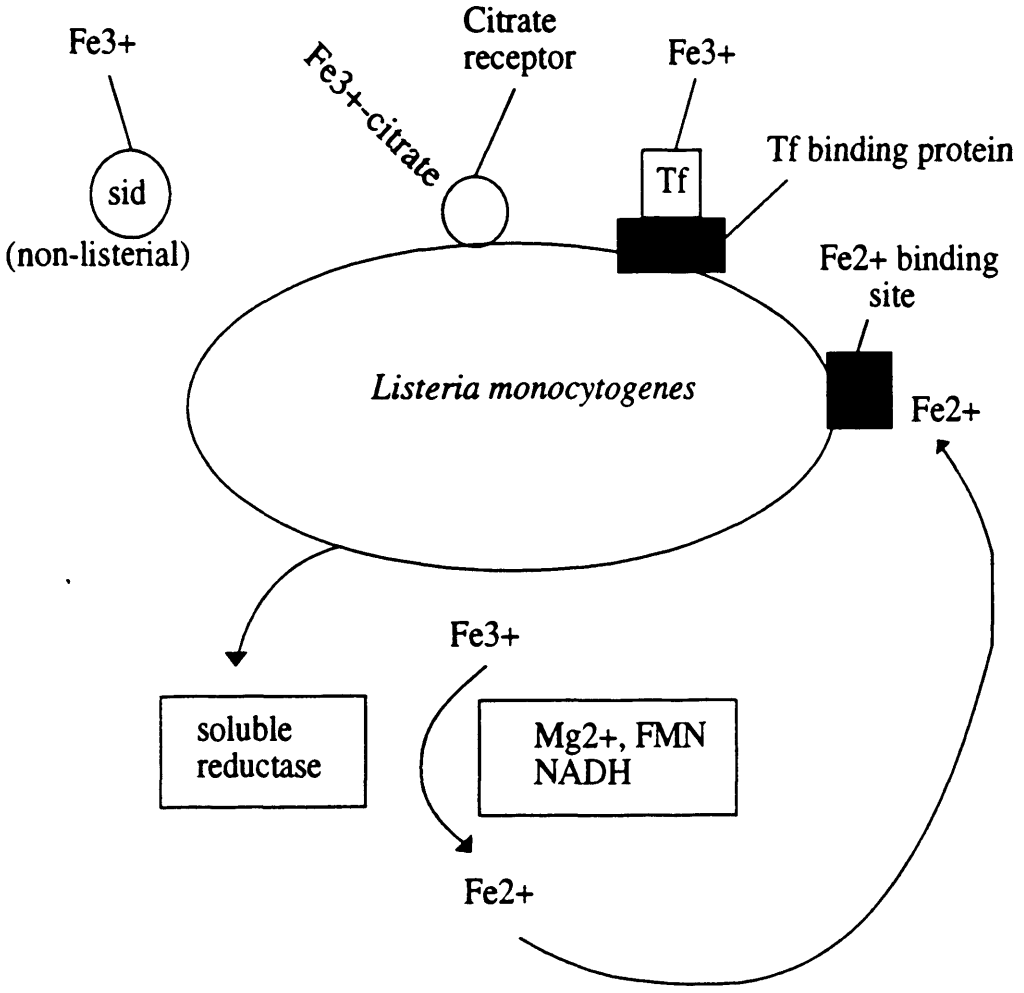
The mechanisms employed by *L. monocytogenes* for acquiring iron appear to be siderophore-independent. Cowart & Foster (1985) made extensive attempts to determine whether *L. monocytogenes* produced either hydroxymate or phenolate type siderophores. Using modifications of standard techniques for siderophore identification (Csaky, 1948; Arnow, 1937) they were unable to detect any siderophores either in the bacterial culture supernates or by analysing extracts of whole cells and concluded that *L. monocytogenes* did not produce siderophores (Cowart & Foster, 1985). A recent study, however, reported the ability of *L. monocytogenes* to utilise iron bound to a variety of iron-chelating compounds, including "foreign" bacterial siderophores (Simon *et al.*, 1995). In this study, it was reported that iron-starvation of *L. monocytogenes*, achieved by growing the cells on agar treated with the iron chelator tropolone (Vidon & Spreng, 1992), caused them to utilise iron bound to a variety of siderophores and catechols. This ability to

utilise iron bound to these compounds was represented by areas of growth on the iron-starved plates in the near vicinity of paper disks containing the siderophores or catechols (Simon *et al.*, 1995). It was suggested that this ability to utilise iron bound to "foreign" siderophores accounted for the ubiquitous nature of *L. monocytogenes*.

Other mechanisms of iron acquisition by this organsim include: (1) production of a soluble ferric reductase, (2) utilisation of ferric citrate via a cell-surface citrate receptor and (3) production of a transferrin binding protein. These mechanisms are represented in Figure 1.3.

In 1985, Cowart & Foster first reported the production of an extracellular reductant from *L. monocytogenes* which was capable of reducing iron bound to transferrin. They followed the ferric reduction by using a ferrous iron chromogenic acceptor, bathophenanthroline sulphonate(BPS) (Cowart & Foster, 1985) and found that slight variation in reductase activity occurred between different strains but the overall activity of the listerial reductant was more rapid than the reduction of ferric-transferrin by dithionite (Kojima & Bates, 1973). A later study suggested that this reductant was a low molecular weight protein of approximately 8-10 kDa which required NADH, flavin mononucleotide (FMN) and Mg^{2+} as co-factors (Cowart *et al.*, 1985). Adams *et al.*, (1990) presented a diagrammatic model of the action of this reductant in listerial iron acquisition (see Figure 1.3). In this model it was proposed that the extracellular reductase donated electrons to FMN from NADH which then reduced ferric iron to ferrous iron. Ferrous iron was then acquired by an energy-independent mechanism, uninhibited by 2, 4- dinitrophenol (DNP) (Adams *et al.*, 1990). The importance of the co-factors for reductase activity was also shown using a biochemical assay (Deneer & Boychuck, 1993; Deneer *et al.*, 1995) which involved the ferrous iron indicator ferrozine (Stookey, 1970). Removal of these co-factors from the assay reaction resulted in a 50% reduction in

Figure 1.3. Iron acquisition mechanisms of *L. monocytogenes*. This representation of listerial iron acquisition is based on the diagram of Adams *et al.*, (1990). Four main mechanisms are indicated: (1) the production of a soluble reductase which requires cofactors, Mg^{2+}, FMN, NADH to reduce Fe^{3+} to Fe^{2+} which is then acquired into the cell via a Fe^{2+} cell receptor, (2) the presence of a transferrin receptor, (3) utilisation of ferric citrate via a citrate receptor and (4) utilisation of siderophores produced by other bacteria. Tf indicates transferrin; sid indicates siderophore.



(Adapted from Adams et al., 1990)

reductase activity (Deneer *et al.*, 1995). This assay also allowed the effect of various environmental conditions on ferric reductase activity to be assessed and it was shown that while iron concentration had no effect on the reductase acitivity of *L. monocytogenes*, low pH and anaerobic conditions greatly reduced the activity and low temperatures increased it (Deneer *et al.*, 1995). The relationship between the reduction of ferric iron and listerial pathogenicity is still unclear, especially as non-pathogenic species have been shown to possess ferric reductase activity (Deneer *et al.*, 1995). However, a molecular biological approach to study this ferric reductase has been adopted (H. G. Deneer, University of Saskatchewan, Personal communication; this study) and the results may lead to a further understanding of the role of the ferric reductase in listerial iron acquisition.

The ability to utilise ferric citrate was demonstrated using radiolabelled [$^{59}Fe^{3+}$]citrate (Adams *et al.*, 1990). The uptake of [$^{59}Fe^{3+}$]citrate by *L. monocytogenes* was shown to be directly related to citrate, as pre-incubation of the cells with citrate led to an inhibition of uptake (Adams *et al.*, 1990). The findings of this work suggested that ferric citrate may be acquired via a citrate receptor, although the exact mechanisms thereafter for transporting the iron into the cell are not known and require further investigations.

The latest mechanism of iron uptake in *L. monocytogenes* to be identified was the production of an 126 kDa transferrin binding protein (Hartford *et al.*, 1993) which would presumably be similar to those produced by the *Pasteurellacae* and *Neisseria* species (Ricard *et al.*, 1990; Archibald & DeVoe, 1979; Norrod & Williams, 1978). The *L. monocytogenes* TBP was not host specific as it could bind transferrins from human, bovine and equine origin. The binding of the transferrins to the listerial protein was visualised using transferrins conjugated to horseradish-peroxidase or alternatively to biotin. However, in a similar study (Bhatt *et al.*, 1994) the workers were unable to

provide evidence to support the existence of the TBP and in contrast their findings suggested that the 126 kDa protein was in fact a naturally biotinylated protein which had an affinity for streptavidin and not transferrin. This aspect obviously requires further investigations to clarify the existence or not of a listerial transferrin binding protein.

Figure 1.3 diagrammatically represents the mechanisms discussed here and provides a simplistic up-to-date model for iron acquisition by *L. monocytogenes*.

1.5 TRANSPOSON MUTAGENESIS OF *L. MONOCYTOGENES*

As mentioned in the previous sections, transposon mutagenesis has proved a useful tool fro identifying and characterising virulence determinants of *L. monocytogenes* (Gaillard *et al.*, 1986; Kathariou *et al.*, 1987; Khun *et al.*, 1990). This relatively recent surge of the use of transposon mutagenesis to study *L. monocytogenes* has been influenced by the successful use of transposons in other Gram positive organisms including, *Streptococcal pyogenes*, *Enterococcus faecalis* and *B. subtilis* (Youngman, 1987) In most studies, banks of transposon mutants are screened for loss of a particular virulence trait and upon identification of the appropriate mutant, the mutated gene can be cloned and the parental gene subsequently isolated and studied (Finlay & Falkow, 1989; Macrina, 1984).

The first report of the use of transposon mutagenesis in *L. monocytogenes* (Gaillard *et al.*, 1986) employed the conjugative transposon *Tn*1545 from *E. faecalis* and this same transposon was later used by other workers (Cossart, 1988; Leblond *et al.*, 1989) to study the listeriolysin O and catalase of *L. monocytogenes*. Another transposon from *E. faecalis*, *Tn*916, which is very similar to *Tn*1545, has also been used successfully to mutagenize *L. monocytogenes* (Kathariou *et al.*, 1987; Sun *et al.*, 1990). Both these transposons are self transmissible and transfer from *E. faecalis* to *L. monocytogenes* by

conjugation which can be achieved using basic mating techniques (Gaillard *et al.*, 1986). Insertion of these conjugative transposons into the listerial chromosome requires homologous sequences between the ends of the transposon and the sequences surrounding the integral site (Clewell *et al.*, 1988), however, this requirement often results in transposon insertion at certain chromosomal regions referred to as hotspots which ultimately reduces the randomness of insertion and so increase the number of mutants necessary to represent a complete transposon library.

To overcome this problem of integration at hotspots on the chromosome, the non-conjugative transposon *Tn*917, also from *E. faecalis*, has been used to study various listerial genes (Camilli *et al.*, 1990; Cossart *et al.*, 1989). The integration of this transposon into the chromosome is much more random in nature and integration is not site specific (Youngman *et al.*, 1983). *Tn*917 also has the advantage of being much smaller (5 kilo bases [kb]) than *Tn*1545 (25.3 kb) or *Tn*916 (16.4 kb) which ultimately simplifies cloning and constructing a map of the chromosomal DNA flanking the insertion. Thus, by exploiting these new generation of transposons (Cossart *et al.*, 1989; Camilli *et al.*, 1990; Youngman, 1987) a better understanding of listerial iron acquisition mechanisms may be achieved.

AIMS

The aim of this investigation was to examine iron acquisition in *L. monocytogenes* using molecular biology techniques. The investigation consisted of two main areas of research:

(1) To study the listerial ferric reductase and identify the encoding gene(s).

(2) To identify gene(s) important for the growth of *Listeria* in low-iron environments.

The first aspect of the investigation was to involve the construction of a listerial genomic library in *E. coli* and the screening for clones expressing ferric reductase activity. A colourimetric assay was available for this purpose. The assay was also to be used for screening a library of transposon mutants of *L. monocytogenes* for any which lacked ferric reductase activity. It was hoped that identification of a ferric reductase encoding gene would provide the starting point for future investigations of the ferric reductase and its role in iron acquisition in *L. monocytogenes*.

The second aspect of the investigation was to identify gene(s) which were important for the growth of *L. monocytogenes* in low-iron environments. The reason for aiming to identify such genes was that they may have encoded proteins which were important for the survival of *Listeria* in low-iron *in vivo*-like environments. A library of transposon mutants of *L. monocytogenes* was to be screened for mutants with deficiencies in their ability to grow in low-iron environments. The low-iron medium which was to be used for screening the library was to be defined in the investigation. It was hoped that identification of mutants with an inability to grow in low-iron environments would provide the starting point for investigating further, the mechanisms by which *L. monocytogenes* acquire iron both *in vitro* and *in vivo*.

MATERIALS & METHODS

2.1 Bacterial strains and growth conditions

The strains used throughout this study are listed in Table 2.1 together with information regarding the source of each strain and distinguishing characteristics.

All *L. monocytogenes* strains were routinely cultured at 37°C in tryptose soya broth (TSB; Oxoid) or agar (TSA; Oxoid) supplemented where appropriate with antibiotics (Sigma). Erythromycin (10 μg/ml), lincomycin (25 μg/ml) and tetracycline (20 μg/ml) were added to the media for growth of *L. monocytogenes* DP-L910 while erythromycin and lincomycin were supplemented for growth of *L. monocytogenes* 7D and 9E. The wild type strain *L. monocytogenes* 10403S had no antibiotic requirements.

While investigating the ferric reductase of *L. monocytogenes*, a modified form of TSA was used. This is discussed in more detail below, in section 2.8 During selection of iron-related insertion mutants from a *Tn*917-pLTV3 listerial library the minimal medium of Trivett & Meyer (1971) and modified versions of this were used. Unmodified Trivett & Meyer medium (1 l) consists of 4 salt solutions (A: K_2HPO_4, 8.5 g, $NaH_2PO_4.2H_20$, 1.7g, NH_4Cl, 500 mg; B: nitrolotriacetic acid, 480 mg, NaOH, 240 mg; C: $FeCl_3$, 29 mg; D: $MgSO_4.7H_20$, 410 mg), amino acids (L-cysteine, L-leucine [100 mg], DL-isoleucine, DL-valine, DL-methionine, L-arginine.HCl, L-histidine.HCl [200 mg]), vitamins (riboflavin, 1 mg, thiamine.HCl, 1 mg, D-biotin, 100 μg, thioctic acid, 10 μg) and glucose (2 g). Modified versions of this medium are described in more detail in section 2.9 below.

When examining the haemolytic activity of *L. monocytogenes* it was necessary to grow the bacteria on TSA containing 5% (v/v) sheep's blood. The appropriate antibiotics were supplemented for observing the haemolytic activity of *L. monocytogenes* 7D and 9E.

Table 2.1. List of Bacterial Strains

STRAIN	GENOTYPE/CHARACTERISTIC	SOURCE/REFERENCE
L. monocytogenes		
10403S	wild type strain	D. Portnoy (University of Pennsylvania)
DP-L910	10403S containing pLTV3	D. Portnoy
7D	iron-related mutant	This study
9E	iron-related mutant	This study
E. coli		
DH5α	*supE44* Δ*lacU169* (Ø80*lacZ*Δ*M15*) *hsdR17 recA1 end*A1 *gyrA96 thi-1 relA1*	Hanahan, (1983)
JM109	F' *traD36 lacI*^{q}Δ(*lacZ*)*M15 proA*$^+$*B*$^+$/ e14$^-$(McrA$^-$) Δ(*lac-proAB*) *thi gyrA96* (Nal^r) *endA1 hsdR17* ($r_k^-m_k^+$) *relA1 supE44 recA1*	Yanisch-Perron *et al.* (1985)
HB101	F' Δ(*gpt-proA*)*62 leuB6 supE44 ara-14 galK2 lacY1* Δ(*mcrC-mrr*) *rpsL20* (Str^r) *xyl-5 mtl-1 recA13*	Raleigh & Wislon, (1986)
MC1061	F$^-$ *araD139* Δ(*ara-leu*)*7696 galE15 galK16* Δ(*lac*)*X74 rpsL* (Str^r) *hsdR2* ($r_k^-m_k^+$) *mcrA mcrB1*	Wertman *et al.* (1986)

Escherichia coli strains were cultured at 37°C in Luria broth (LB: 10 g tryptone; 5 g yeast extract; 5 g sodium chloride [NaCl]) with the addition of 1.5% (w/v) agar (LA) as required. When necessary, antibiotics were added to the media - kanamycin (20 μg/ml for the growth of *E.coli* strains containing pJM1 or pJM2; ampicillin (100 μg/ml) for the growth of *E.coli* strains containing pTTQ18, pBluescript or pJM6, pJM7 and pJM9.

2.2 Plasmids

A list of the plasmids used or constructed during this study can be seen in Table 2.2. Plasmids pTTQ18 and pBluescript^{SK} where obtained from departmental stocks while pLTV3 was obtained from Dr D. Portnoy, University of Pennsylvania.

All plasmids designated pJM were constructed during the course of this study and are described in more detail in the Results sections which follow.

2.3 DNA extraction and purification

During this study, small and large scale extraction of various plasmids were performed. In addition, isolation of chromosomal DNA from *L. monocytogenes* was carried out regularly.

2.3.1. Small scale plasmid extraction

Plasmid extractions were performed as described by Sambrook *et al.* (1989). Stationary phase bacterial cells (1.5 ml) were harvested by centrifugation at 13,000 rpm for 1 min in an MSE Microcentaur bench top microfuge. The pellet was resuspended in 100 μl of ice-cold Solution I (0.01 M diaminoethanetetra-acetic acid disodium salt [EDTA; pH 8.0]; 2% (w/v) glucose; 0.05 M Tris-HCl [pH 8.0]) containing 10 mg/ml

Table 2.2. List of Plasmids

PLASMID	CHARACTERISTICS	REFERENCE
PTTQ18	High copy number *E.coli* vector; ; ampicillin resistance; blue-white selection	Stark, (1987)
pBluescript^{SK}	As above	Short *et al.*, (1988)
pLTV3	Carrrier of Tn917::lac fusion (see Figure 3.15)	Camilli *et al.* (1990)
pJM1	Based on pLTV3; contains 5.5 kb listerial DNA	This study
pJM2	Based on pLTV3; contains 0.5 kb listerial DNA	This study
pJM6	pBluescript containing 6 kb listerial DNA	This study
pJM7	pBluescript containing 0.8 kb listerial DNA	This study
pJM9	pBluescript containing 0.7 kb listerial DNA	This study

lysozyme. Following incubation on ice for 30 min, 200 μl of Solution II (0.2M sodium hydroxide [NaOH]; 1% (w/v) sodium dodecyl sulphate [SDS]) was added and incubation continued for a further 5 min before the addition of 150 μl of ice-cold Solution III (11.5% (v/v) acetic acid; 3 M potassium acetate). After incubating, on ice for 20 min, the cell debris was removed by centrifugation at 13,000 rpm for 5 min in a Microcentaur microfuge. Phenol:chloroform extraction of the 400 μl supernate was performed by the addition of 400 μl phenol:chloroform (1:1) followed by vigorous mixing using a bench vortex (Gallenkamp). Centrifugation at 13,000 rpm for 5 min in a Microcentaur microfuge was performed. After a single chloroform extraction, the DNA was precipitated from the aqueous phase by the addition of 0.1 volumes of 3 M sodium acetate (pH 5.2) and 2.5 volumes of 100% ethanol. After a 30 min incubation period at -20°C, the DNA was recovered by centrifugation at 13,000 rpm for 20 min in a Microcentaur microfuge, dissolved in 50 μl of sterile nanopure H_2O and stored at -20°C until required.

2.3.2 Large scale plasmid extraction

2.3.2.1 Polyethylene glycol (PEG 8000) precipitation

For large scale plasmid extractions (Sambrook *et al.*, 1989), 400 ml of stationary phase cells were harvested by centrifugation at 8,000 rpm for 10 min at 4°C in a Sorval RC-5B centrifuge, Sorval GSA rotor. The pellet was resuspended in 10 ml of ice-cold solution I and incubated, on ice, for 30 min. Solution II was added (20 ml), mixed gently, and incubated for a further 10 min on ice, followed by the addition of 15 ml of ice-cold Solution III and a further 10 min incubation and centrifugation at 16,000 rpm for 30 min at 4°C in a Sorval RC-5B centrifuge, Sorval SS-34 rotor. The DNA was precipitated by the addition of 12 ml of isopropanol and after an incubation period of 15 min at room temperature (25°C) the DNA was recovered by centrifugation at 8,000 rpm for 20 min at 20°C in a Sorval RC-5B centrifuge and dissolved in 3 ml of TE buffer

(Tris - EDTA buffer: 1 mM EDTA [pH 7.8]; 10 mM Tris-HCl [pH 8.0]). High-molecular-weight RNA was precipitated from the DNA by the addition of 3 ml of ice-cold 5 M lithium chloride and centrifuged at 10,000 rpm for 10 mins at 4°C in a Sorval RC-5B centrifuge. The remaining DNA in the supernate was again precipitated using isopropanol (12 ml) and recovered by centrifugation at 10,000 rpm for 10 min at 20°C. After washing the DNA pellet with 70% (v/v) ethanol, it was dissolved in 500 μl of TE buffer (pH 8.0) containing DNase-free pancreatic RNAase (20 μg/ml) and following incubation at room temperature for 30 min, 500 μl of 1.6 M NaCl containing 13% (v/v) PEG 8000 was added, mixed well and plasmid DNA recovered by centrifugation at 13,000 rpm for 5 min at 4°C in a Microcentaur microfuge. The pellet of plasmid DNA was dissolved in 400 μl of sterile nanopure H_2O and extracted once with Tris-saturated phenol (Fisons), once with phenol:chloroform (1:1) and once with chloroform as described in the previous section. The DNA was precipitated from the aqueous phase by adding 0.1 volumes of 3 M sodium acetate (pH 5.2) and 2.5 volumes 100% ethanol. After a 30 min incubation at -20°C the DNA was recovered by centrifugation at 13,000 rpm for 20 min in a Microcentaur microfuge. The pellet was dissolved in 500 μl of sterile nanopure H_2O and stored at -20°C until required. The DNA concentration of the preparation was calculated as described in Section 2.4.3 below.

2.3.2.2 Qiagen column extraction of plasmid DNA

An alternative procedure for purifying large amounts of plasmid DNA was to use Qiagen Maxi columns (Qiagen Ltd, UK) and the protocol provided with the kit. Essentially, 500 ml of stationary phase cells were harvested by centrifugation at 6,000 rpm for 15 min at 4°C in a Sorval RC-5B centrifuge, GSA rotor, and the resulting pellet was resuspended in 10 ml of Resuspension Buffer P1 (50 mM Tris-HCl, pH 8.0; 10 mM EDTA) containing 100 μg/ml RNase A. After mixing thoroughly, 10 ml of Lysis Buffer P2 (200 mM NaOH; 1% SDS) was added and incubated at room temperature for 5 min after which 10 ml of Neutralization Buffer P3 (3 M potassium

acetate, pH 5.5) was added and incubation continued, on ice, for 20 min. Cell debris was removed by two centrifuation steps at 13,000 rpm for 30 min at 4°C in a Microcentaur microfuge. Meanwhile a Qiagen-tip 500 was equilibrated with 10 ml of Buffer QBT (750 mM NaCl; 50 mM MOPS, pH 7.0; 15% (v/v) ethanol; 0.15% (v/v) Triton X-100) at room temperature. The supernate was then added to this equilibrated column and allowed to pass through by gravity flow, after which 2 X 30 ml of Wash Buffer QC (1 M NaCl; 50 mM MOPS, pH 7.0; 15% (v/v) ethanol) was passed through the column. The plasmid DNA on the column was then eluted by adding 10 ml of Elution Buffer QF (1.25 M NaCl; 50 mM Tris-HCl, pH 8.5; 10% (v/v) ethanol) to the column and collecting the eluate. The DNA was precipitated from the aqueous phase by adding 7 ml of isopropanol and collected by centrifugation at 11,000 rpm for 30 min at 4°C in a Sorval RC-5B centrifuge, SS-34 rotor, washed once with 70% (v/v) ethanol before being resuspended in 2 ml of sterile nanopure H_2O. DNA concentration was calculated as in section 2.4.3.

2.3.3 Chromosomal DNA extraction

The method used for extracting chromosomal DNA from *L. monocytogenes* was essentially as described by Flamm *et al.* (1984). An overnight bacterial culture (10 ml TSB) was harvested by centrifugation at 13,000 rpm for 10 min at 4°C in a Sorval RC-5B centrifuge, SS-34 rotor, and washed twice with 5 ml of 0.1X SSC (1X SSC: 0.15 M NaCl; 0.015 M trisodium citrate [pH 7.0]). The resulting pellet was resuspended in 1 ml of 0.01 M sodium phosphate buffer (pH 7.0) in 20% (w/v) sucrose containing 2.5 mg/ml lysozyme (Sigma) and incubated for 45 min at 37°C. Following this, 9 ml of 1% (w/v) SDS was added together with 1 mg/ml proteinase K and incubation continued for 30 min. This solution was repeatedly phenol:chloroform (1:1) extracted until no white protein precipitate formed at the interface. Ten millilitres of phenol:chloroform were added and mixed thoroughly before centrifugation at 4,000 x g for 15 min at 4°C in a Hereaus low-speed centrifuge. The DNA was precipitated from the aqueous layer

by the addition of 0.1 volume of sodium acetate (pH 5.2) and 2.5 volumes ice-cold ethanol, collected by spooling onto a glass rod, air-dried and dissolved in 1 ml of sterile nanopure H_2O. The DNA was stored at -20°C until required.

2.3.3.1 Size selection of chromosomal DNA fragments

For construction of the partial genomic libraries, 2-5 kb and 6-9 kb chromosomal DNA fragments were required. After digestion with *Sau*3A, a maximum of 200 ng of DNA were loaded onto a 13.5 ml sucrose gradient. The gradient was formed by freezing a solution of 20% (w/v) sucrose in buffer (20 mM Tris-HCl [pH 8.0]; 1 M NaCl; 5 mM EDTA [pH 8.0]) for at least 2 hrs and then thawing overnight at 4°C. After the gradient had formed, the DNA was added, centrifuged at 26,000 rpm for at least 16 hrs at 4°C in an OTD Combi Ultracentrifuge using a Sorval, AH-527 swing-out rotor. The tube was punctured and the gradient was dripped into approximately 30 x 0.5 ml aliquots and every third aliquot analysed by gel electrophoresis. After determining which aliquots contained the appropriate DNA fragments, they were pooled together and dialysed overnight in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) at 4°C, ethanol precipitated and dissolved in 30 μl of sterile nanopure H_2O. The concentration of DNA was calculated as described in section 2.4.3.

2.4 Routine DNA manipulations

2.4.1 Agarose gel electrophoresis

Separation of DNA fragments of different sizes involved electrophoresis on agarose gels. The concentration of agarose (SeaKem) used depended on the size of the fragments being separated. 0.7% (w/v) agarose in TAE buffer (40 mM Tris-acetate; 1 mM EDTA; pH 7.8) was used to separate fragments greater that 3 kb, while fragments less that 3 kb were separated by 1% (w/v) agarose gel electrophoresis. Electrophoresis

was performed in TAE buffer containing 0.5 μg/ml ethidium bromide. DNA samples and molecular weight markers (1 kb ladder, or bacteriophage lambda DNA digested with *Hind*III or *Xho*I, [Gibco BRL]) were mixed with loading buffer (0.25% (w/v) bromophenol blue; 0.28% (w/v) xylene cyanol FF; 15% (w/v) Ficoll Type 400 [Pharmacia]) prior to electrophoresis and visualised afterwards using a long-wave ultraviolet transilluminator.

2.4.2 Extraction of DNA from agarose gels

2.4.2.1 Sephaglas Bandprep Kit (Pharmacia)

Essentially, the extraction was performed as described in the manufacturers instruction booklet. The band of interest was excised from the gel and dissolved in Gel Solubiliser solution (sodium iodide buffered with Tris-HCl [pH 8.0]) at 65°C for 5 min. Sephaglas BP was added (5 μl/μg DNA) and continually mixed on a rotary wheel for 5 min at room temperature. The DNA bound to the Sephaglas BP was collected by centrifugation at 13,000 rpm for 15 seconds in a Microcentaur microfuge and washed twice in Wash solution (20 mm Tris-HCl, [pH 8.0]; 1mM EDTA; 0.1 mM NaCl; 18% (v/v) ethanol). The DNA was eluted from the air-dried Sephaglas pellet using 15 μl of Elution buffer (10 mM Tris-HCl, [pH 8.0]; 1 mM EDTA) and stored at -20°C until required.

2.4.2.2 Low melting point agarose (LMP)

An alternative method used for extracting DNA from agarose gel slices was the use of LMP agarose as described by Sambrook et al. (1989). DNA samples (1 to 2 μg) were separated through 0.7% (w/v) LMP (Gibco, BRL) agarose in TAE buffer and visualised using a long wavelength UV transilluminator. The band(s) of interest were excised and added to 5 volumes of nanopure H_2O. After an incubation of 5 min at

65°C, the solution was extracted once with Tris-HCl saturated phenol, once with phenol:chloroform (1:1) and once with chloroform. The DNA was precipitated by the addition of 0.1 volume 3M sodium acetate (pH 5.2) and 2.5 volumes of ethanol (100%) and collected by centrifugation at 13,000 rpm for 20 min at 4°C in a Microcentaur microfuge. The DNA pellet was dissolved in sterile nanopure H_2O and stored at -20°C until required.

2.4.3 Calculating DNA concentration and purity

DNA concentration was calculated as described by Sambrook *et al.* (1989). For a known volume of DNA, the optical density at 260 nm (OD_{260}) was recorded and the value obtained was applied to the following equation to give DNA concentration as μg/ml:

$$OD_{260} \times 50 \times \text{dilution factor}$$

where 50 represents a conversion factor based on 50 μg/ml of double stranded DNA giving an OD_{260} of 1. The purity of the DNA was also checked by recording the OD_{280} of the DNA sample. A ratio OD_{260}:OD_{280} of 1:8 suggests the DNA sample is pure while a ratio of less than 1:8 is suggestive of protein or phenol contamination.

2.4.4 Endonuclease restriction of DNA

All restriction endonucleases used in this study were obtained from Gibco (BRL) and cleavage of the DNA was performed according to the supplier's recommendations. Essentially, 0.5 to 1 unit of enzyme was used to digest 500 ng of DNA in a reaction volume of 20 μl. Digestion was allowed to continue for 1 hr at 37°C before inhibition of enzyme reaction either by heating to 65°C or by phenol:chloroform extraction.

2.4.5 Dephosphorylation of plasmid DNA

Endonuclease digested plasmid DNA was often required to be dephosphorylated. Calf intestinal alkaline phosphatase (CIAP) was obtained from Promega and dephosphorylation performed as described in the manufacturer's leaflet. Plasmid DNA (10 pmoles of 5' termini) was treated with 0.1 unit of CIP using the reaction buffer provided by the supplier. Following a 30 min incubation at 37°C a further 0.1 unit of CIP was added and incubation continued for a further 30 min at 37°C. The reaction was stopped by phenol:chloroform extraction and the DNA precipitated as described previously in section 2.3.1.

2.4.6 DNA ligation

DNA ligation was carried out at room temperature for 24 hrs using T4 DNA Ligase (Gibco BRL) and the accompanying ligase buffer. The amounts of vector and insert used varied between ligations but were determined essentially as described by Sambrook et al. (1989). In certain areas of the study, an alternative method for DNA ligation was used. However, this is discussed in more detail in Section 3.5.

2.5 Transformation of *E. coli* by electroporation

2.5.1 Preparation of electrocompentent cells

The method used for electrocompetent cell preparation was described by Dower *et al.* (1988). A fresh overnight culture (10 ml LB) was used to inoculate 1 L of LB and incubation was continued at 37°C with vigorous shaking until the cells reached mid-logarithmic phase (OD_{600} 0.5 to 1.0). The cells were then chilled on ice for 30 min before harvesting by centrifugation at 6,000 rpm for 15 min at 4°C in a Sorval RC-5B centrifuge, GSA rotor. The resulting cell pellet was resuspended in ice-cold sterile

nanopure H_2O and centrifuged at 6,000 rpm for 20 min at 4°C in a Sorval RC-5B centrifuge, SS-34 rotor. This step was repeated twice more and was followed by one wash with ice-cold 10% (v/v) glycerol. The final cell pellet was resuspended in 1 ml of ice-cold 10% (v/v) glycerol and the competent cells were stored in 40 μl aliquots at -70°C until required but for no longer than 6 mths.

2.5.2 Electro-transformation of *E. coli*

Aliquots of electrocompetent cells were thawed on ice immediately before use and to the 40 μl of cells, 2 μl of DNA (0.2 μg) were added and mixed. These were then added to an ice-cold 0.2 cm^3 electroporation cuvette (BioRad) and placed in the safety chamber of the BioRad Gene Pulser and Pulse Controller apparatus (Dower *et al.*, 1988). After adjusting the setting to 25 μF, 2.5 kV and 200 Ω, the cells were given a single pulse and allowed to recover by the addition of 1 ml of SOC medium (2% (w/v) tryptone, 0.5% (w/v) yeast extract; 10 mM NaCl; 10 mM magnesium chloride, 10 mM magnesium sulphate, 20 mM glucose, 2.5 mM potassium chloride) followed by an incubation period of 1 hr at 37°C. Transformants were obtained by plating onto selective agar.

2.6 Construction of a partial genomic library of *L. monocytogenes* 10403S

Genomic DNA fragments from *L. monocytogenes* 10403S were cloned into the vector pTTQ18 and transformed into *E.coli* to create a listerial genomic library in *E.coli*. The chromosomal DNA from *L. monocytogenes* was partially digested with *Sau*3A to give maximum fragments either in the 2-5 kb or 6-9 kb region and after purification by using the Bandprep Kit (BioRad) or a sucrose density gradient (see Section 2.3.3.1 and 2.4.2) were ligated into pTTQ18 using T4 DNA Ligase.

The vector was *Bam*HI restricted and dephosphorylated using CIP. The efficiency of dephosphorylation was examined by comparing the electrophoretic profiles of phosphorylated and dephosphorylated DNA which had previously been incubated overnight at room temperature with T4 DNA Ligase. Transformation of this self-ligated phophorylated and dephosphorylated plasmid DNA into *E. coli* was also performed and the numbers of transformant obtained were compared.

Following ligation of the chromosomal DNA fragments of either 2 -5 kb or 6 - 9 kb into the *Bam*HI-restricted dephosphorylated pTTQ18, plasmid mini preparation were performed on 12 random transformants to examine the overall percentage of total transformants which possessed chromosomal DNA inserts. The plasmid DNAs were digested with *Bam*HI and the electrophoretic profiles obtained were compared with the profile of *Bam*HI restricted pTTQ18.

2.7 Transposon mutagenesis of *L. monocytogenes*

Transposon mutagenesis of *L. monocytogenes* was performed using the method described by Camilli *et al.*, (1990). *L. monocytogenes* 10403S (DP-L910) containing the plasmid pLTV3 was grown at 42°C for 24 to 48 h on TSA with erythromycin, lincomycin at concentrations described in section 2.1. This induced transposition of the *Tn*917 derivative from pLTV3 into the chromosome of the listeria. The frequency and randomness of insertion of *Tn*917 into the listerial chromosome was assessed by A. L. Passos (Department of Microbiology, University of Leicester) and the results obtained are discussed in more detail section 3.1.

2.8 Screening for the listerial ferric reductase

The biochemical assay used to screen both the genomic library and the transposon library of mutants for the listerial ferric reductase was first described in *L.*

monocytogenes by Deneer & Boychuck (1992). The assay utilises the ferrous iron chelator, ferrozine (3-[2-pyridyl]-5-6, bis-[4-phenyl sulfonic acid] -1,2,4 -triazine; Sigma) which was described by Stookey (1970). Binding of ferrous iron, the reduced product of ferric iron, to the chelator, results in a change in colour from white to deep red in the medium surrounding the bacterial colony.

2.8.1 Screening the listerial genomic library for a ferric reductase-positive *E. coli* clone

When screening the listerial genomic library for an *E.coli* transformant possessing reductase activity, the transformants were patched onto a master plate and a test plate and incubated overnight at 37°C. The master plate and the test plate were essentially TSA which had been treated with conalbumin A (Sigma) to reduce the levels of free iron (Gutteridge, 1987). After autoclaving the modified TSA, 100 μg/ml of ferric ammonium citrate (Sigma), 20.8 mM ethylenediamine-di-(o-hydroxyphenylacetic acid) [EDDHA; Sigma] and 100 μg/ml; ampicillin were added to both media. In addition, 20 μg/ml tetracycline and 25 μg/ml kanamycin were added to the master plate medium while 10 μg/ml of IPTG (Sigma) were added to the test plate medium. The test plate was used for the ferric reductase assay while the master plate was used as a reference source of the clones examined and was maintained at 4°C. Bacteria inoculated onto both plates were incubated at 37°C for 24 h.

Any bacterial growth on the test plate was overlain with 10 ml of indicator mix consisting of 0.5% agarose, 10 mM magnesium chloride, 15 μM flavin mononucleotide (Sigma) and 2 mM ferrozine. The overlain plate was incubated at 37°C for 1 to 4 hrs during which time the appearance of a deep red colour surrounding the colony was visible if reductase activity was present.

The assay was used initially to test for the presence of reductase activity in wild type

strain *L. monocytogenes* 10403S and also to confirm the reductase negativity of the *E. coli* strain DH5α, the strain into which the listerial DNA was transformed. In the screening of the library, 100 individual transformants were patched onto each test plate. The negative control, *E. coli* DH5α containing pTTQ18 was also plated onto this test plate, while the positive control *L. monocytogenes* 10403S was plated onto the same medium but without the addition of ampicillin.

2.8.2 Screening the transposon library of *L. monocytogenes* mutants

The ferric reductase assay was used to detect a ferric reductase negative mutant from a library of transposon mutants of *L. monocytogenes* (section 2.7) The procedure for screening was essentially the same as that used for screening the genomic library, although in this case we were looking to identify a *L. monocytogenes* mutant which lacked the ferric reductase activity and thus would appear white instead of deep red on the test plate. The test medium was as described above except for the addition of erythromycin and lincomycin to select for the transposon-containing listerial mutants.

2.9 Screening for transposon mutants defective in growth in low iron

The transposon library of *L. monocytogenes* mutants described in section 2.7 was also used to identify mutants with a deficiency in their ability to survive in environments with minimal iron levels. Essentially, a low-iron media were identified and inoculated with individual *L. monocytogenes* mutants and their growth examined. These media were modified forms of the medium of Trivett & Meyer (1971). The normal level of ferric chloride (178 μm) in TM was ommitted and replaced with 1 μm ferric chloride ($FeCl_3$) or 1 μm ferrous sulphate ($FeSO_4$) and the resulting media were designated TM.Fe^{3+} and TmFe^{2+}, respectively. These altered media provided the low-iron environment in which the screening for mutants with a deficiency in ability to survive in low-iron was to be performed. More detail, and a diagrammatic representation of this

screening procedure are given in section 3.2.

2.10 DNA hybridisation

DNA hybridisation were performed throughout this study. For example, DNA hybridisation was used (1) to confirm transposon insertion in *L. monocytogenes* mutants, (2) to construct restriction maps of various DNA fragments and (2) to confirm the existence of chromosomal DNA from *L. monocytogenes* 10403S in various plasmids.

2.10.1 Transfer of DNA to nylon filters by Southern blotting

DNA samples were separated on 0.6% (w/v) agarose gels as described previously (Section 2.4.1) and photographed alongside a linear ruler. Southern blotting of the DNA from the gel to the nylon membrane was performed as described by Southern (1975). The DNA in the gel was partially depurinated by soaking in 300 ml of 0.25 M HCl for 7 min. After rinsing in distilled H_2O the gel was placed in 300 ml of denaturing solution (0.5 M NaOH, 1.5 M NaCl) and soaked for 30 min, after which it was rinsed in distilled H_2O and placed in 300 ml neutralising solution (0.5 M Tris-HCl [pH 7.5], 1.5 M NaCl, 1 mM EDTA) for 30 min. A platform for the gel was formed inside a tray containing 20X SSC (3 M NaCl, 0.3 M trisodium citrate [pH 7.5]) and consisted of a gel casting plate covered with 3 sheets of Whatman 3 mm filter paper soaked in 20X SSC. The gel was placed on this platform and covered with the nylon membrane (BDH) and 3 sheets of Whatman 3 mm paper which had all been previously soaked in 3X SSC. On top of this arrangement a stack of paper towels and a 300 g weight placed were placed and transfer was allowed to proceed overnight at room temperature. After transfer was complete the nylon membrane was removed, air dried and the DNA fixed to the membrane by exposing to UV light for 2-3 min. The membrane was wrapped in clingfilm and stored at 4°C until required.

2.10.2 Generating a radio-labelled DNA probe

DNA to be used to probe the DNA fixed to the nylon membrane was radioactively labelled by random priming using the Pharmacia Ready-To-Go labelling kit. The lyophilised reaction components (dATP, dTTP, dGTP, Klenow fragment of DNA polymerase I and random 9-base long oligomers) were reconstituted in 20 μl of sterile nanopure H_2O for 10 min before the addition of approximately 50 ng of probe DNA.

Probe DNA was denatured by boiling for 5 min and placed on ice for 5 min prior to mixing with the reaction mix. Immediately, 3 μl of [α-^{32}P] dCTP (10 μCi; Amersham International) was added to this reaction mix containing the probe DNA and the total volume made up to 50 μl with sterile nanopure H_2O and incubated at 37°C for 30 min to 2 hrs. Before use, the radiolabelled probe DNA was denatured, as before, by boiling and cooling on ice.

2.10.3 DNA-DNA hybridisation

All hybridisations in this study were performed under conditions of high stringency (Sambrook *et al.*, 1989). A pre-hybridisation step was performed for 2 hrs at 65°C prior to hybridising with the radiolabelled probe. Twenty five milliliters of pre-hybridisation solution (5X SSC; 5X Denhardt's solution [50X Denhardt's: 1% (w/v) Ficoll Type 400; 1% (w/v) bovine serum albumin; 1% polyvinylpyrolidone]; 0.5% (w/v) SDS; 100 μg/ml denatured salmon sperm DNA) was added to the membrane and incubated at 65°C for 2 hrs. Following this, the radiolabelled probe was added and incubation continued for at least 12 hrs. Three high stringency washes were performed by incubating the probed membrane in 0.1X SSC/0.1%[w/v] SDS at 65°C for 15 min each time, after which the membrane was air dried, wrapped in clingfilm and placed inside a developing cassette (Hybaid) alongside Cronex film (Dupont) for 4 to 24 hrs.

Films were developed in an Agfa-Geveart automatic film processor.

2.10.4 Stripping radiolabelled probes from nylon membranes

Occassionally, membranes were stripped of the radiolabelled probe to enable re-probing of the fixed DNA with a different probe. Firstly, the membrane was washed with 0.1X SSC at room temperature for 10 min. Three 15 min washes at 65°C with 1% (w/v) SDS followed and the efficiency of the striping procedure was examined by exposing x-ray film to the membrane to confirm for the absence of radiolabel.

2.11 Polymerase chain reaction (PCR)

In general, PCR reactions were based on the method of Saiki *et al.*, (1988). Reactions were carried out in 50 μl volumes containing, 100 - 500 ng of template DNA, 1 μM of each primer, 200 μM deoxynucleotides, X1 Taq polymerase buffer (20 mM Tris-HCl, pH 8.3, 25 mM KCl, 0.05% (v/v) Tween 20), 3 mM $MgCl_2$ and 1 U Taq polymerase (Gibco, BRL). The reaction were overlaid with 30 μl mineral oil (molecular grade, Sigma) and placed into a Perkin-Elmer model, 480, Thermal cycler to allow the PCR to occur. The parameter of the thermal cycler were, 1 cycle at 94°C, 2 min; 30-40 cycles at 94°C, 1 min, 55-60°C, 1 min, 72°C, 1 min; and 1 cycle at 72°C, 4 min. The PCR reactions were stored at -20°C until required for analysis by gel electrophoresis.

A list of the primers used throughout this study is given in Table 2.3. Further descriptions of the primers designed in this study are given in sections, 3.6 and 3.7 below. The primers were purchase from Gibco, BRL.

2.12 DNA sequencing

DNA sequencing was performed by automated sequencing using the PRISM Dyedeoxy

Cycle Sequencing Kit on Model 373A Sequence System (Applied Biosystems).

2.12.2 Automated DNA sequencing

Sequencing was carried by The Nucleic Acid DNA Sequencing Laboratory, University of Leicester, using the ABI 373A DNA automated sequencer (Applied Biosystems). In the sequencing reactions double-stranded DNA template was used at a final concentration of 100-500 ng/μl. Approximately 3.2 pmole of primer was used in a cycle sequencing reaction which consisted of 25 cycles of 96°C, 30 sec, 50°C, 15 sec and 60°C, 4 min. The ABI PRISM™ Dye Terminator Cycle Sequencing kit (Perkin Elmer) was used in conjunction with the automated sequencer. This kit used dye-labelled terminators to carry out enzymatic extension reactions for DNA sequencing. The enzyme used is AmpliTaq DNA Polymerase FS (Applied Biosystems) which is a modified version of Taq DNA polymerase. The dNTP mix used during extension reactions included dITP rather than dGTP to minimise band compressions, which may occur due to the stable base-pairing in GC-rich regions. The reaction products were extracted once with choloroform to remove the mineral oil and ethanol precipitate. The resulting product was washed twice in 70% (v/v) ethanol prior to analysis at The Nucleic Acid Sequencing Laboratory, University of Leicester.

2.13 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

2.13.1 Preparation of crude membrane fractions

Crude membrane fractions of *L. monocytogenes* were prepared as described by Belyi *et al.*, (1992). Bacteria were grown for 18 h at 37°C in 50 ml volumes of TSB, with shaking at 180 rpm. The cells were pelleted by centrifugation in a Sorval RC-5B

Table 2.3. Table of oligonucleotide primers

PRIMER	SEQUENCE (5'-3')	SIZE	TM(°C)	ORIGIN
ERM.PROX	GCAATAACCGTTACCTGTTTGTGCC	25	62	This study
ERM.DIST	GGCCTTGAAACATTGGTTTAGTGGG	25	61	This study
FLAA	GCAGCAACTGTAGAACCACTACCTA	25	59	This study
CHEV1	TCGGCTTACTTACTTGTGCGTCGGC	25	63	This study
CHEV2	GAAAATATGTTGCTTGGGCTTCGCG	25	58	This study
TN917-HI5	ACGAAAATAACTAAACTCGCTTGGC	25	62	This study
T3	AATTAACCCTCACTAAAGGG	20		Short *et al.* (1988)
T7	GTAATACGACTCACTATAGGGC	22		As above
ARGC1	GCCTGGATTGGCAATGAGTTTCGC	24		This study
ARGC2	TAAAGTTTTGCACTGCCTGACCGGC	25		This study

centrifuge, GSA rotor at 8,000rpm for 20 min at 4°C and washed once in 20 mM Tris-HCl (pH 7.8). Bacterial cells were then resuspended in 5 ml of Tris- HCl (pH 7.8) and lysed by sonication (MSE sonicator, 4-5 μm amplitude, 20kHz). Unbroken cells were removed by centrifugation in a Sorval RC-5B centrifuge, GSA rotor, at 8,000 rpm for 30 min at 4°C and the supernate was centrifuged at 22,000 rpm for 60 min at 4°C to pellet the cell membrane. The resulting pellet was resuspended in Tris-HCl (pH 7.8) supplemented with 1 mM EDTA, 150 mM NaCl and 15 (v/v) Triton X-100. After incubation for 1 h at 37°C the suspension was centrifuged in a Sorval RC-5B centrifuge, SS-34 rotor at 22,000 rpm, for 60 min at 4°C and the supernate was used as the crude membrane preparation. The proteins were quantified using the method of Markwell (1978) and were solubilised in sample buffer (125 mM Tris.HCl [pH 6.8], 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, 0.002% (w/v) bromophenol blue) by boiling for 5 min.

2.13.2 Electrophoresis and staining

Protein samples were separated by SDS-PAGE using the SDS discontinuous system of Laemmli (1970). Briefly, 20 μl (20 μg) of protein were loaded into each well of a 5% (w/v) acrylamide stacking gel (1.7 ml 29% (w/v) acrylamide/1% (w/v) bisacrylamide solution, 1.25 ml 1 M Tris-HCl pH6.8, 100 μl 10% (v/v) SDS, 100 μl 10% (w/v) ammonium persulphate, 10 μl TEMED, per 10 ml) and separated in a 12% (w/v) acrylamide resolving gel (20 ml 29% (w/v) acrylamide/1% (w/v) bisacrylamide solution, 12.5 ml 1 M Tris-HCl pH6.8, 500 μl 10% (v/v) SDS, 500 μl 10% (w/v) ammonium persulphate, 20 μl TEMED, per 50 ml). Electrophoresis was carried out in a vertical slab gel apparatus (Bio Rad) in a buffer comprising 25 mM Tris-HCL, 192 mM glycine and 0.1% (w/v) SDS (pH8.3), at a constant current of 20 mA per gel through the stacking gel and 30 mA per gel through the resolving gel. Proteins were visualised by staining for 2-4 h with 0.1% (w/v) Coomassie Brilliant Blue (Sigma) in 10% (v/v) acetic acid and 45% (v/v) methanol and destaining for approximately 2 h

with several changes of the destaining solution (7.5% (v/v) acetic acid; 5% (v/v) methanol).

2.14 Measurement of β-galactosidase activity from *L. monocytogenes*

The level of β-galactosidase expressed from *L. monocytogenes* 10403S, 7D and 9E was examined using the method of Miller (1972). The standard procedure involved growth of the bacterium to an OD_{600} of 0.4. 0.1 ml of culture was mixed with 0.9 ml Z buffer (60 mM $Na_2HPO_4.7\ H_2O$, 40 mM $NaH_2PO_4.H_2O$, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, pH 7.0), 2 drops of chloroform and 1 drop of 0.1% (w/v) SDS and incubated at 30°C for 5-10 min. *o*-nitrophenyl-β-D-galactopyranoside (ONPG; 0.2 ml of 4 mg/ml solution) was added and incubation at 30°C was allowed to continue until a yellow coloured developed, when the reaction was stopped by the addition of 0.5 ml 1 M $NaCO_3$-. The absorbency at 550 and 420 nm was recorded and used to calculate the units of β-galactosidase activity with the following equation:

$$\frac{1000 \times A_{420} - (1.71 \times A_{550})}{t \times 0.1 \times A_{600}}$$

where A_{420}, A_{550} are the absorbencies of the reaction, A_{600} is the OD_{600} of the culture used for the assay and t is the time between when the reaction was started by the addition of ONPG and stopped by the addition of 1 M $NaCO_3$-. The condition which were used for growing the bacteria being assayed varied and are discussed in more detail in sections 3.6 and 3.8 below.

2.15 Electron microscopy of flagella from *L. monocytogenes*

The bacteria being used for electron microscopic examination of the flagella were

grown under various condition, as described in section 3.8 below. Electron microscopy was kindly performed by Robert Gilbert (Department of Microbiology, University of Leicester). 10 μl of the cell samples were placed on a carbon-coated grid and washed in 10 μl distilled water before staining for 15 sec with unbuffered uranyl acetate. The grids were visualised using a Joel 100 CX transmission electron microscope using an accelarating voltage of 100 kV. The calculated magnification for each micrograph is given in the figure legends in section 3.8 below.

RESULTS

3.1 Studies of the ferric reductase of *L. monocytogenes*

The goal of the work described in this section was to clone the gene(s) involved in the ferric reductase activity expressed by *L. monocytogenes*. The cloned gene(s) could then be used as the starting point for investigations into the role of ferric reductase in iron acquisition by *L. monocytogenes*.

Two main strategies were undertaken: (1) construction and screening of a listerial genomic library for a clone expressing ferric reductase activity, (2) screening of a library of *Tn*917 listerial mutants for a clone lacking ferric reductase activity.

3.1.1 Ferric reductase assay

The assay used to screen both libraries was a colourimetric assay based formation of a deep red colour when ferrous iron chelator, ferrozine, was complexed to ferrous iron (Dailley & Lascelles, 1971). The assay is shown diagrammatically in Figure 3.1a.

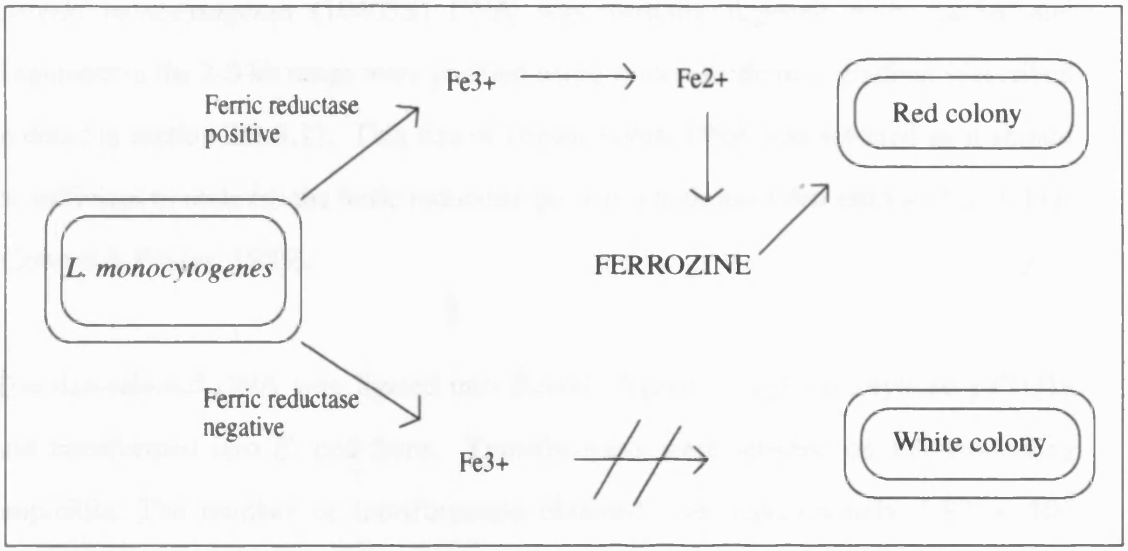
Initially, the assay was used to confirm the ferric reductase-positive phenotype of the wild type strain, *L. monocytogenes* (10403S) and likewise to confirm the reductase-negative phenotype of the *E. coli* strain Sure used for cloning the listerial library. The results are shown in Figure 3.1B were the marked difference in colour between the ferric reductase-positive *L. monocytogenes* 10403S (A1) and the ferric reductase-negative *E. coli* Sure (B) is evident. *L. monocytogenes* produced a deep red colour around the colonies while *E. coli* Sure remained white.

Thus, the construction and screening of the listerial genomic library was undertaken.

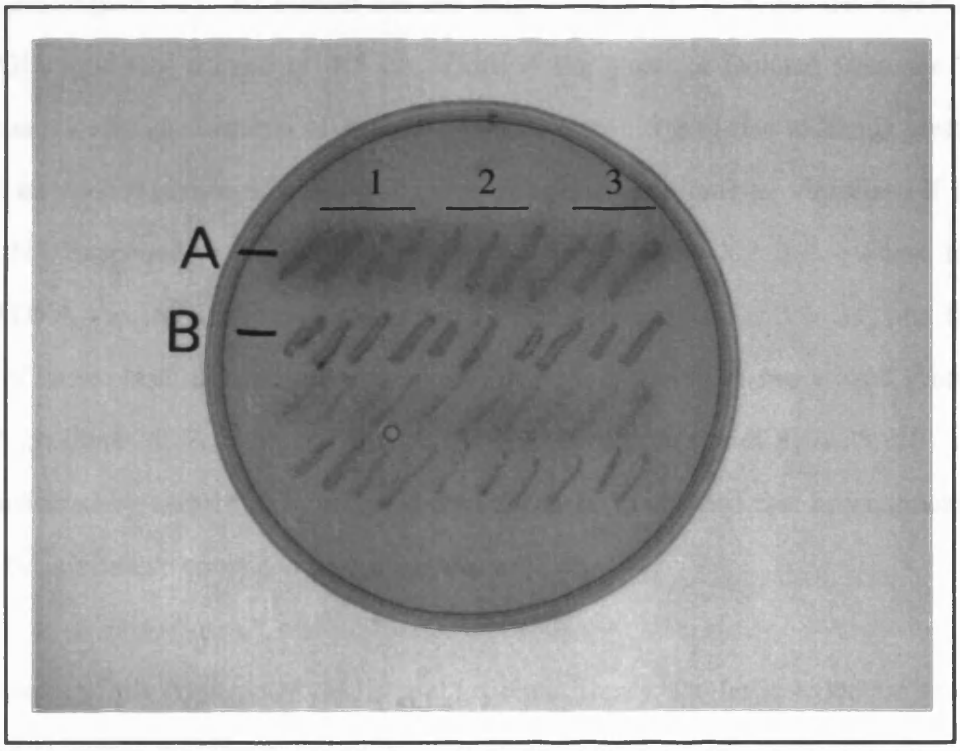
Figure 3.1a. Diagrammatic representation of the ferric reductase assay. Simple diagram showing the ferric reductase assay used for screening the listerial libraries for the ferric reductase encoding gene. The assay was based on the formation of a deep red colour when the ferrous iron (Fe^{2+}) chelator, ferrozine, was complexed with Fe^{2+} (Stookey, 1970) and was described initially for Listeria by Deneer & Boychuck (1993).

Figure 3.1b. Photograph showing ferric reductase activity. Ferric reductase activity of *L. monocytogenes* 10403S (A1) and *E. coli* Sure (B) as examined using the assay described by Deneer & Boychuck (1993). Colonies labelled A2 and A3 are discussed in further detail in section 3.3 below.

A


Fe3+
Fe2+
Ferric reductase positive
Red colony
L. monocytogenes
FERROZINE
Ferric reductase negative
Fe3+
White colony

B


1
2
3
A
B

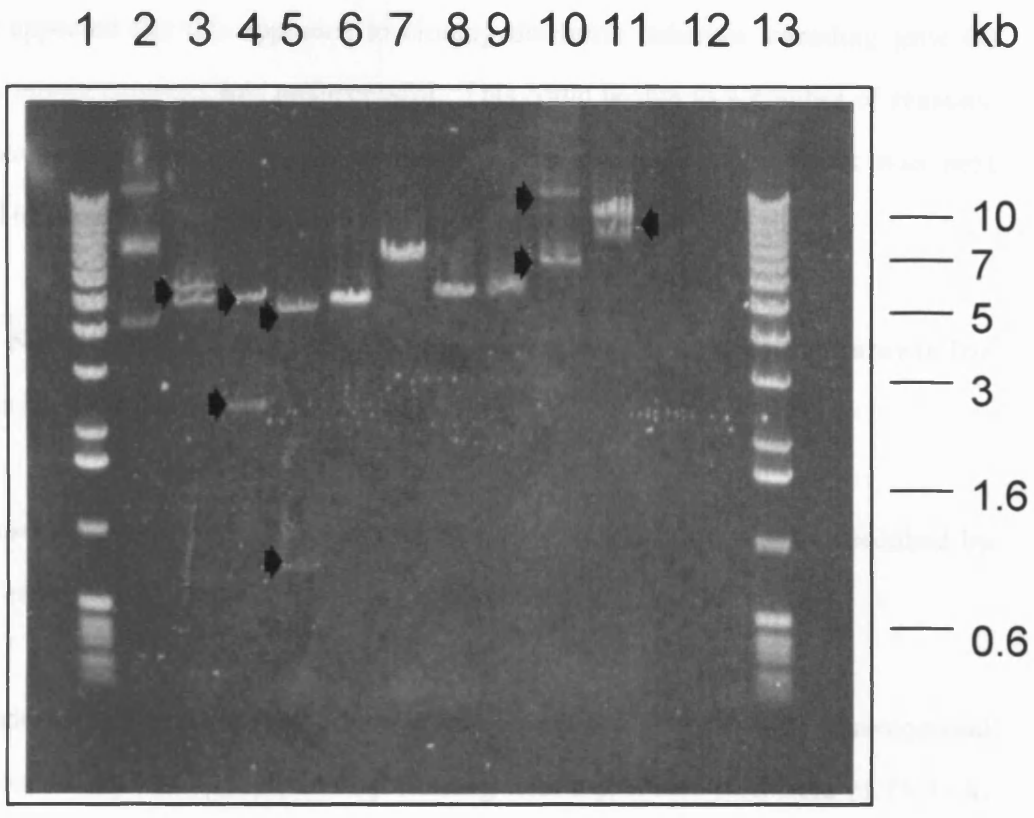
3.1.2 Construction of a listerial genomic library and screening for the ferric reductase

Listeria monocytogenes (10403S) DNA was partially digested with *Sau*3A and fragments in the 2-5 kb range were purified using a sucrose density gradient (described in detail in section 2.3.3.1). This size of chromosomal DNA was selected as it should be sufficient to code for the ferric reductase protein which has been estimated as 9 kDa (Cowart & Foster, 1985).

The size-selected DNA was ligated into *Bam*HI-digested, dephosphorylated pTTQ18 and transformed into *E. coli* Sure. Transformants were selected on LA containing ampicillin. The number of transformants obtained was approximately 1.83 x 10^5 cfu/μg DNA. Plasmid DNA was isolated from 10 random transformants to assess the percentage of transformants containing plasmids with inserts of listerial genomic DNA. After isolation, the plasmids were digested with *Bam*HI and analysed on a 0.7% (w/v) agarose gel (Figure 3.2). As *Bam*HI has one unique site in pTTQ18, *Bam*HI-digestion of pTTQ18 produced a band of 4.5 kb. Thus, if the plasmids isolated from the 10 transformants contained inserts of listerial DNA they would give rise to bands greater than 4.5 kb upon digestion with *Bam*HI. More than one band may be visualised if the insert DNA happened to contain a *Bam*HI site. From Figure 3.2 it is evident that plasmid DNA was isolated from 9 out of the 10 transformants (lanes 3 to 11) and that all nine of these plasmids appeared to have an insert, four giving rise to a band greater than 4.5 kb (lanes 6, 7, 8, 9) and five producing two bands (lanes 3, 4, 5, 10, 11; bands indicated by arrows). From these data it can be concluded that approximately 80% of transformants contained recombinant plasmids.

Having successfully constructed the listerial genomic library, the ferric reductase assay was used to screen the library for an *E. coli* clone showing ferric reductase activity. This would be represented by a deep red colour around the colony.

Figure 3.2. Plasmid profiles of 10 random transformants from a listerial genomic library. Plasmid DNA from 10 library clones was prepared, digested with *Bam*HI, and analysed on a 0.7% (w/v) agarose gel. Lanes 3-12 represent the 10 clones, while lane 2 represents plamsid pTTQ18. Lanes 1 and 13 represent 1 kb ladder.


1 2 3 4 5 6 7 8 9 10 11 12 13
kb
10
7
5
3
1.6
0.6

In excess of 10,000 transformants were screened and none were shown to have ferric reductase activity, all maintaining the white colouration around the colony. The positive control, *L. monocytogenes* 10403S, was included in all screenings and was shown to produce a deep red colour around the colony. The equation of Clark & Carbon (1977) calculates the number of transformants which should be screen to have a 99% probability of identifying the gene of choice. The number of transformants screened was actually in excess of the 8,500 required as predicted by the Clark & Carbon (1977) equation.

Thus it appeared that this approach to cloning the ferric reductase encoding gene (s) from *L. monocytogenes* was unsuccessful. This could be due to a number of reasons, which are considered in detail in section 4. The emphasis of the work was next directed towards screening the library of listerial mutants.

3.1.3 Screening a library of *L. monocytogenes* transposon mutants for a clone lacking ferric reductase activity

Transposon mutagenesis of *L. monocytogenes* 10403S was performed as described by Camilli *et al.*, (1991) and is discussed in more detail in section 2.7.

The randomness of transposon insertion was examined by extracting chromosomal DNA from 12 random mutants and hybridising with a probe derived from pLTV3 (A. L. Passos, Personal communication). The results showed that 9 out of 12 insertions were in different sites of the chromosome while 3 out of the 12 insertions were in the same site of the chromosome. It was concluded that the randomness of insertion was approximately 75%. The ferric reductase assay was used to screen this library for a *L. monocytogenes* clone lacking the ferric reductase activity. An extensive screen was once again carried out, with over 12,000 mutants being examined.

On the initial screen of approximately 12,000 transposon mutants, 11 mutants were identified as ferric reductase-negative because they appeared as white colonies. However, when these mutants were re-screened, they appeared to be ferric reductase positive, producing a red colour around the colony. The reasons for these observations are unknown but are discussed further in section 4.1.

It was felt at this stage of the work that extensive time and energy had been put into the two strategies undertaken to clone the ferric reductase encoding gene(s). Since both had proved unsuccessful, the emphasis the work was switched towards the other aspect of iron acqusition being investigated because this study was proving to be more successful than the ferric reductase investigation.

3.2 Screening a transposon library of *Listeria* mutants for isolates with deficiencies in ability to grow in a low iron environment

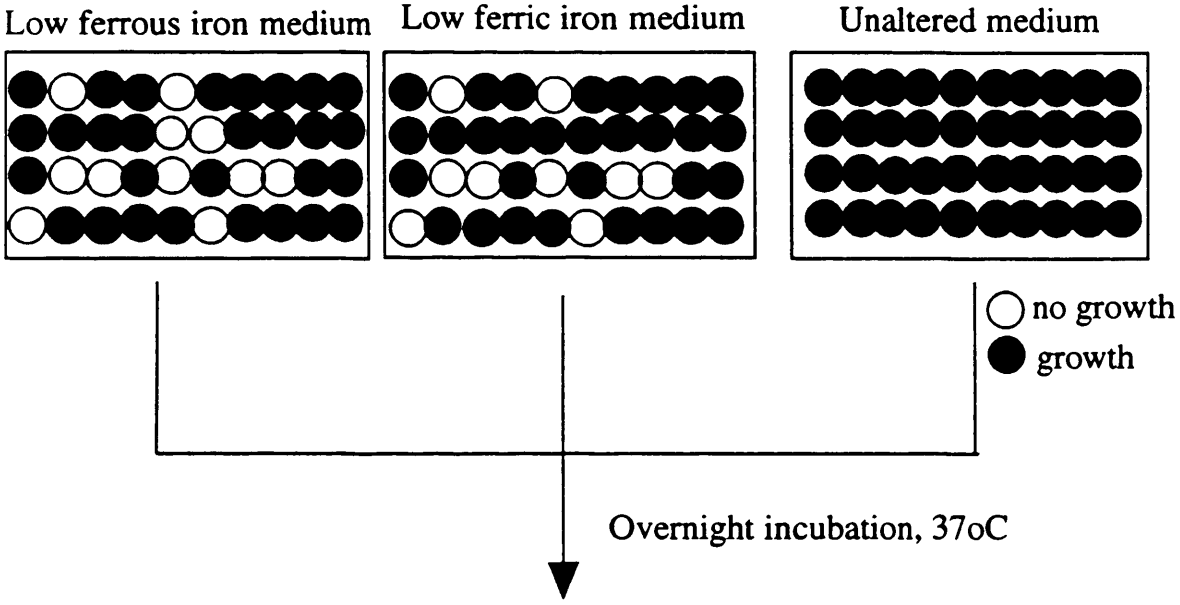
The main objective of this investigation was to identify *L. monocytogenes* transposon mutants with a deficiency in ability to acquire or utilise iron compared with the wild type strain (*L. monocytogenes* 10403S). These were defined as "iron-related" mutants and this term is used throughout this thesis.

The screening procedure is outlined in Figure 3.3. Essentially, each mutant was inoculated into an individual well of three separate 96-well microtitre plates. The microtiter plates contained one of three liquid media: (1) a medium with low levels of ferrous iron, (2) a medium with low levels of ferric iron and, (3) an unaltered (iron-complete) medium. After overnight incubation at 37°C, the growth was assessed by eye and those colonies which showed visibly reduced growth in either or both of the low iron medium but normal growth in the unaltered medium were re-examined. Upon re-examination, if the phenotype was the same, the growth of the mutants were examined in larger volumes of media.

Initially, the possibility of using a complex medium such as TSB for screening the library was examined. This would minimise the likelihood of identifying auxotrophic mutants. However, the problem with using a complex medium was that the amount of ferrous or ferric iron initially present was not known. If in excess it may mask any mutations which were related to the acquisition of iron.

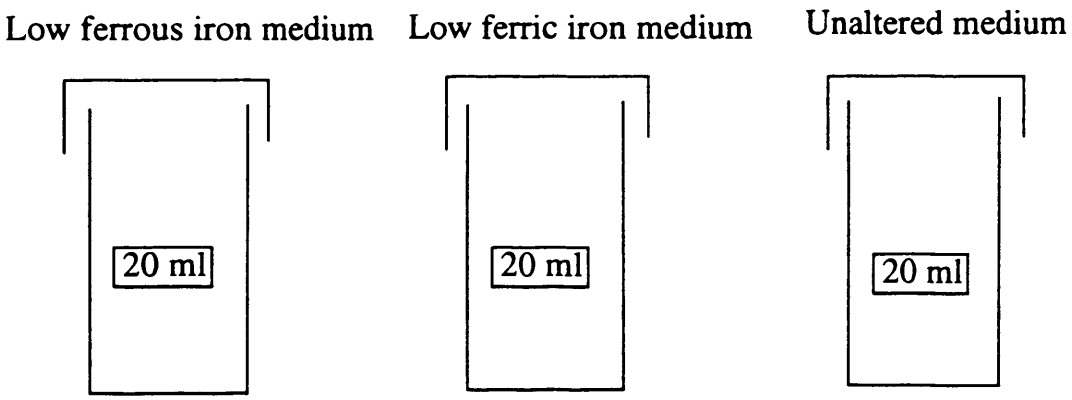
Thus we decided to examine various iron chelators and their ability to remove the iron from TSB. We started by examining EDDHA, because this chelator has been used by other workers in the field to identify iron-related bacterial mutants (Pope *et al.*, 1996; Sokol, 1987; Tai *et al.*, 1993). In this present investigation, iron was removed from TSB using conalbumin A and EDDHA, as described previously by Deneer &

Figure 3.3. Schematic representation of the screening procedure used to identify iron-related mutants from a listerial transposon library. Diagram showing a simple representation of the protocol used for screening the library of listerial transposon mutants for isolates with a deficiency in ability to grow in either or both low iron (ferrous or ferric) media. Initial screens were performed in 96-well microtiter plates and the level of bacterial growth was examined by eye. Confirmatory screens were performed in 20 ml volumes of medium and the level of growth was examined spectrophotometrically (OD_{600}).



Overnight incubation, 37oC

Rescreened, as above, isolates with visibly reduced growth in either or both low iron media but normal growth in unaltered media.



Finally, rescreen in large volumes of media, overnight incubation, 37oC, shaking.

Record the OD600 at 24 hr.

Boychuck (1993). Although, four times as much chelator as that used by Deneer & Boychuck (1993) was required to to see any significant inhibitory effect on listerial growth. Even when this amount (10.4 mM) was added, complete inhibition of growth was never observed. EDDHA was toxic above this level. In Figures 3.4A and 3.4B the toxic effect of EDDHA on the growth of *L. monocytogenes*, 10403S can be seen. When less than 10.4 mM EDDHA was added to the medium, no apparent effect on the growth was observed (Figure 3.2A and 3.4B). Concentrations between 10.4 mM and 20.8 mM inhibited the growth in a manner which could be reversed by the addition of 100 μM ferrous sulphate ($FeSO_4$) (Figure 3.4A) or 100 μm ferric chloride ($FeCl_3$) (Figure 3.4B) Inhibition of growth by 41.6 mM EDDHA was reversed by addition of $FeSO_4$ but not $FeCl_3$. EDDHA was toxic above this level. Concentrations of 60, 70 and 83.2 mM EDDHA inhibited the growth in a manner which could not be reversed by the addition of 100 μm $FeSO_4$ or $FeCl_3$

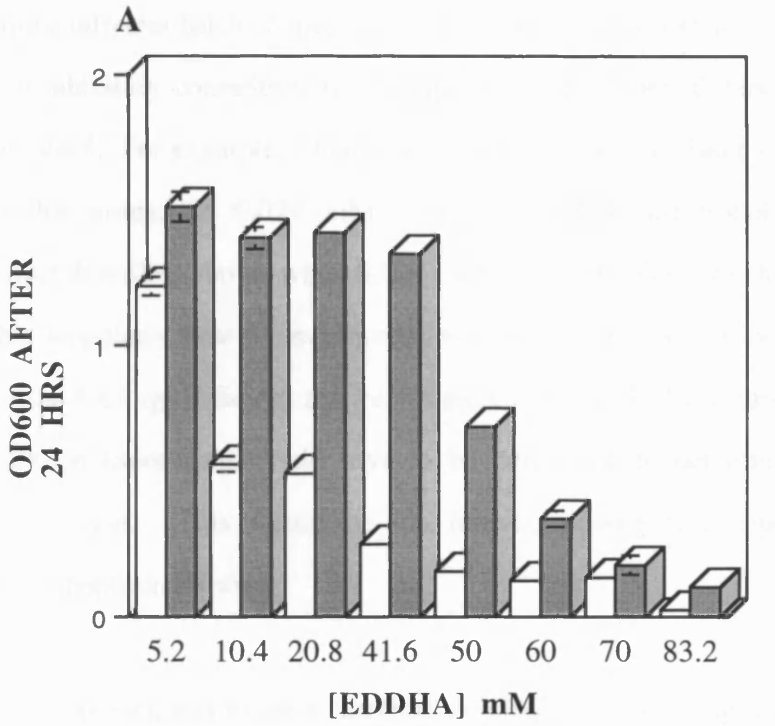
As is evident from Figures 3.4A and 3.4B, growth was visible in medium treated with 10.4 mM EDDHA even when no additional ferrous or ferric iron was added, reaching an OD_{600} ranging from 0.3 to 0.5. This presented a problem if we were going to use this medium for screening the transposon library for iron-related mutants, because the screening procedure was going to involve the observation, by eye, of growth or no growth in the wells of microtiter plates; this residual growth would not make this a straightforward exercise.

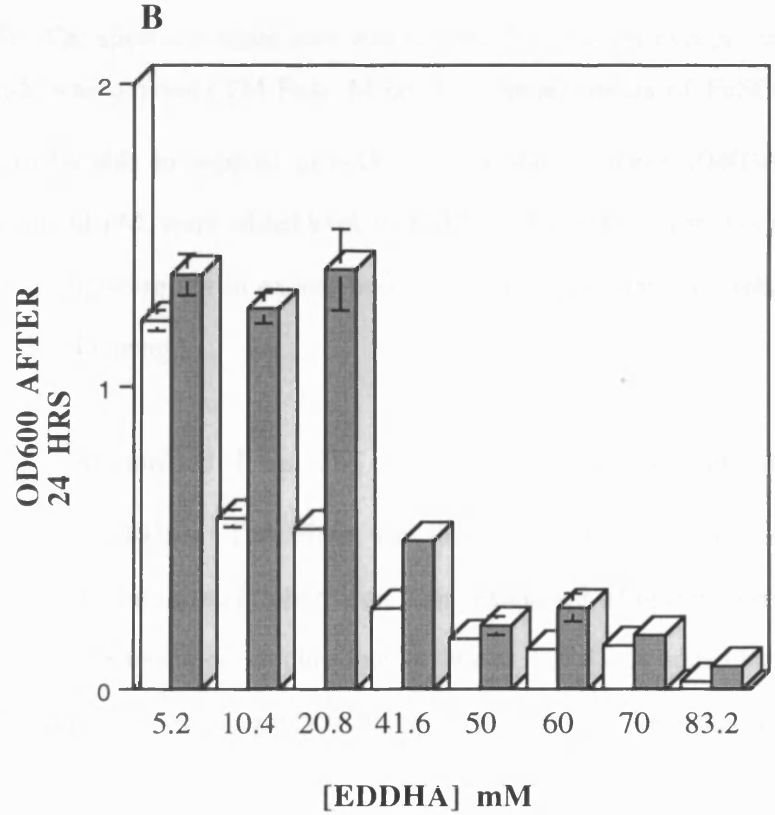
At this point another iron chelator was examined; namely tropolone (Vidon & Spreng, 1992). The recent report of Vidon & Spreng, (1995) suggested that tropolone was much more efficient at removing iron from TSB than other iron chelators including, 2, 2 - dipyridyl, deferoxamine and ethylene diamine tetraacetate (EDTA).

The initial experiment examining this chelator produced data which were promising. We found that 0.024 mM of tropolone when added to TSB (Batch A) completely

Figure 3.4. Effect of EDDHA concentration in TSB on listerial growth

The OD_{600} *L. monocytogenes* 10403S after incubation for 24 hr at 37°C, shaking, in TSB containing various amounts of EDDHA. Cultures where grown in the presence (grey columns) or absence (white columns) of added iron. Ferrous sulphate ($FeSO_4$) was used as the iron source in graph (A) while ferric chloride ($FeCl_3$) was used as the iron source in graph (B). The results represent the mean value of 3 experiments and the standard error of the mean (SEM) is shown by the vertical bars.


A
OD600 AFTER 24 HRS
2
1
0
5.2
10.4
20.8
41.6
50
60
70
83.2
[EDDHA] mM

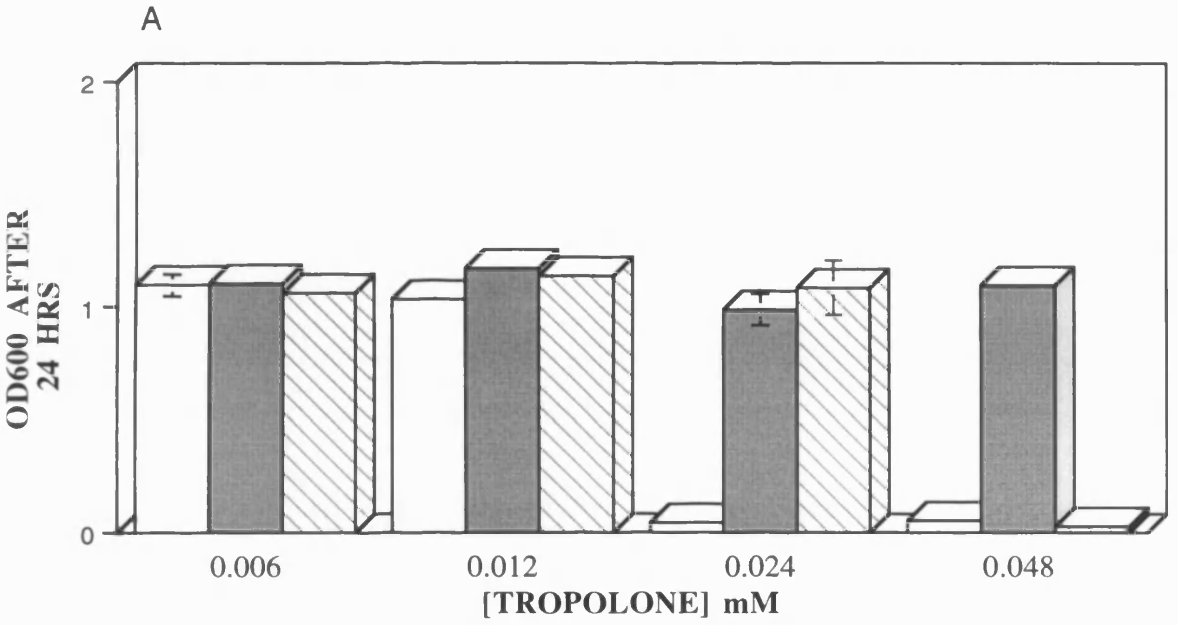

B
OD600 AFTER 24 HRS
2
1
0
5.2
10.4
20.8
41.6
50
60
70
83.2
[EDDHA] mM

inhibited the growth of *L. monocytogenes* 10403S, although the subsequent addition of 100 μM $FeSO_4$ or $FeCl_3$ reversed this inhibitory effect and the growth was similar to that observed in untreated TSB (Figure 3.5A). However, when this experiment was repeated using a different batch of media (Batch B), the results obtained suggested that the minimum inhibitory concentration of tropolone varied when different batches of medium were used. For example, when a new batch of medium (Batch B) was used it was shown that instead of 0.024 mM tropolone, 0.048 mM tropolone gave the inhibitory effect described above, while 0.024 mM did not inhibit growth at all (Figure 3.5B). This tropolone titration experiment was performed on various batches of medium and the findings indicated that each time a new batch of medium was used, a tropolone titration experiment would have to be performed to determine the correct concentration to use. This would be too time-consuming and impractical when screening the transposon library.

Thus, the next approach was to use a chemically defined medium, despite the possibility of identifying auxotrophic mutants. Trivett & Meyer medium (TM), was used because this was reported recently as being the best defined medium for listerial growth (Jones *et al.*, 1995). The approach taken here was to use TM medium except that the 178 μM ferric chloride was omitted (TM-Fe). Minimum concentrations of $FeSO_4$ and $FeCl_3$, determined to be able to support growth of *L. monocytogenes* 10403S to the same extent as unaltered TM, were added back to TM-Fe. The reason for this was to ensure that the iron levels were not in excess and would not mask any iron related mutations which might be occuring.

When $FeCl_3$ was omitted from TM we saw an inhibition of growth of *L. monocytogenes*, 10403S. The effect on listerial growth of addition of various concentrations of ferrous and ferric iron was examined (Figures 3.6A and 3.6B, respectively). This experiment implies that as little as 1 μM iron added back to the iron-depleted TM supports growth similar to that in unaltered TM. Less than 1 μM ferric or

Figure 3.5. Effect of tropolone concentration in TSB on *listerial* growth
The OD_{600} of *L. monocytogenes* 10403S after incubation for 24 hr at 37°C, shaking, in Batch A (3.5A) or Batch B (3.5B) of TSB containing various concentrations of tropolone. Either no iron (white columns), 100 μM $FeSO_4$ (grey columns), or 100 μM $FeCl_3$ (hatched columns) was added to the tropolone-treated medium. These results are the mean values of four different sets of data. The SEM is shown by the vertical bars.


A
2
1
0
OD600 AFTER 24 HRS
0.006
0.012
0.024
0.048
[TROPOLONE] mM

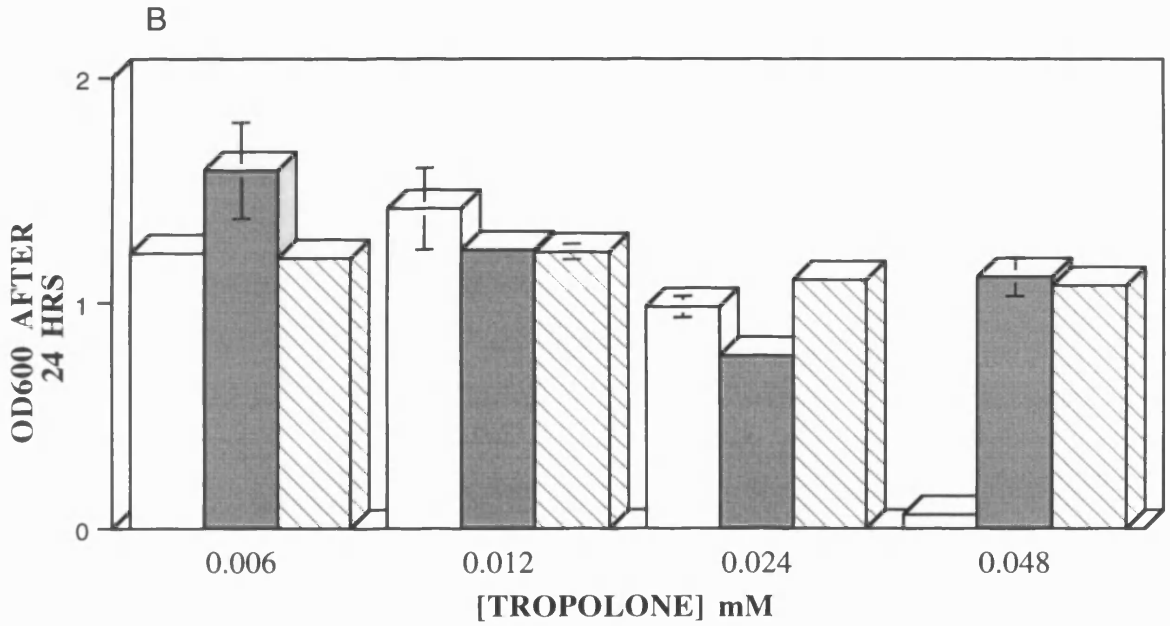
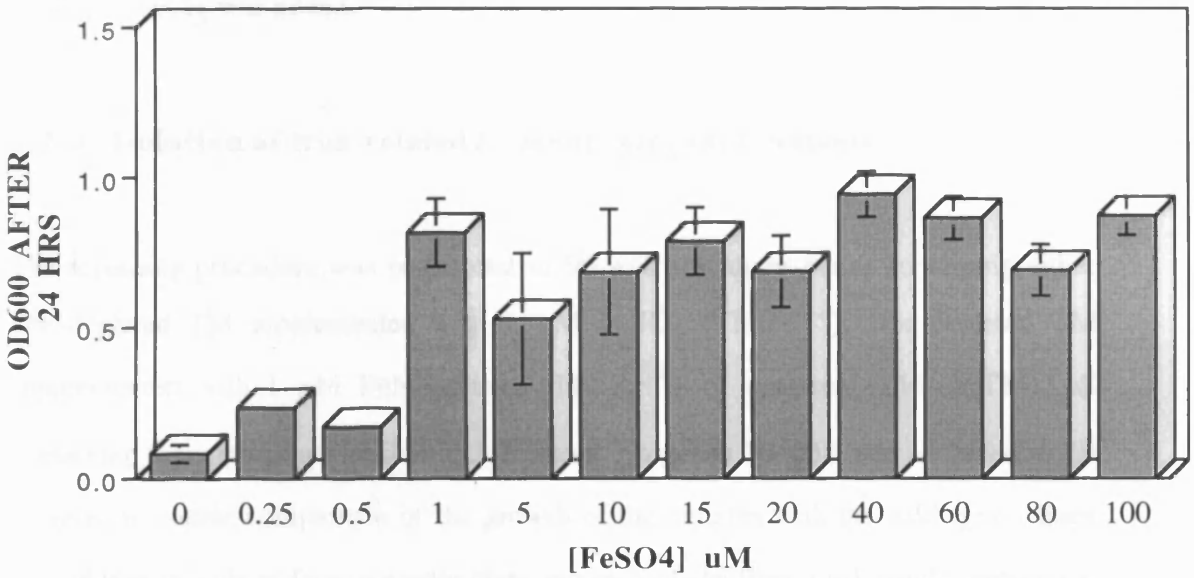

B
2
1
0
OD600 AFTER 24 HRS
0.006
0.012
0.024
0.048
[TROPOLONE] mM

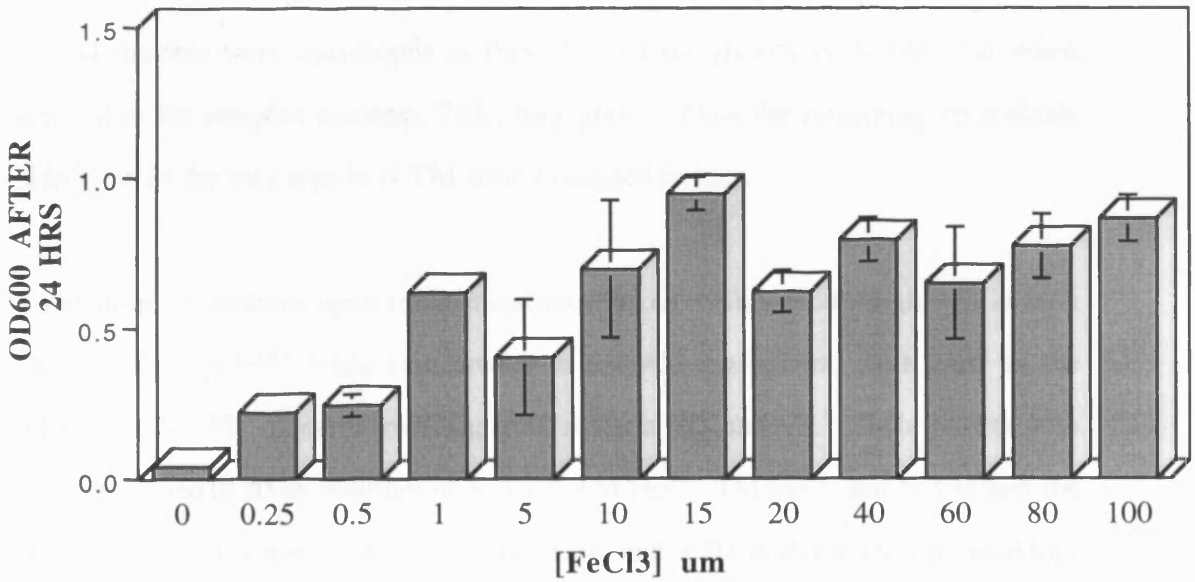
Figure 3.6. Effect of iron concentration of TM on *listerial* growth

The OD_{600} of *L, monocytogenes* 10403S after incubation for 24 hr at 37°C, shaking, in iron-depleted TM to which 0, 0.025, 0.5 1, 5, 10, 15, 20, 40, 60, 80, and 100 μM $FeSO_4$ (A), or $FeCl_3$ (B) was added. These results represent the mean values of four different sets of data and the SEM is shown by the vertical bars.

A


OD600 AFTER 24 HRS
1.5
1.0
0.5
0.0
0
0.25
0.5
1
5
10
15
20
40
60
80
100
[FeSO4] uM

B


OD600 AFTER 24 HRS
1.5
1.0
0.5
0.0
0
0.25
0.5
1
5
10
15
20
40
60
80
100
[FeCl3] um

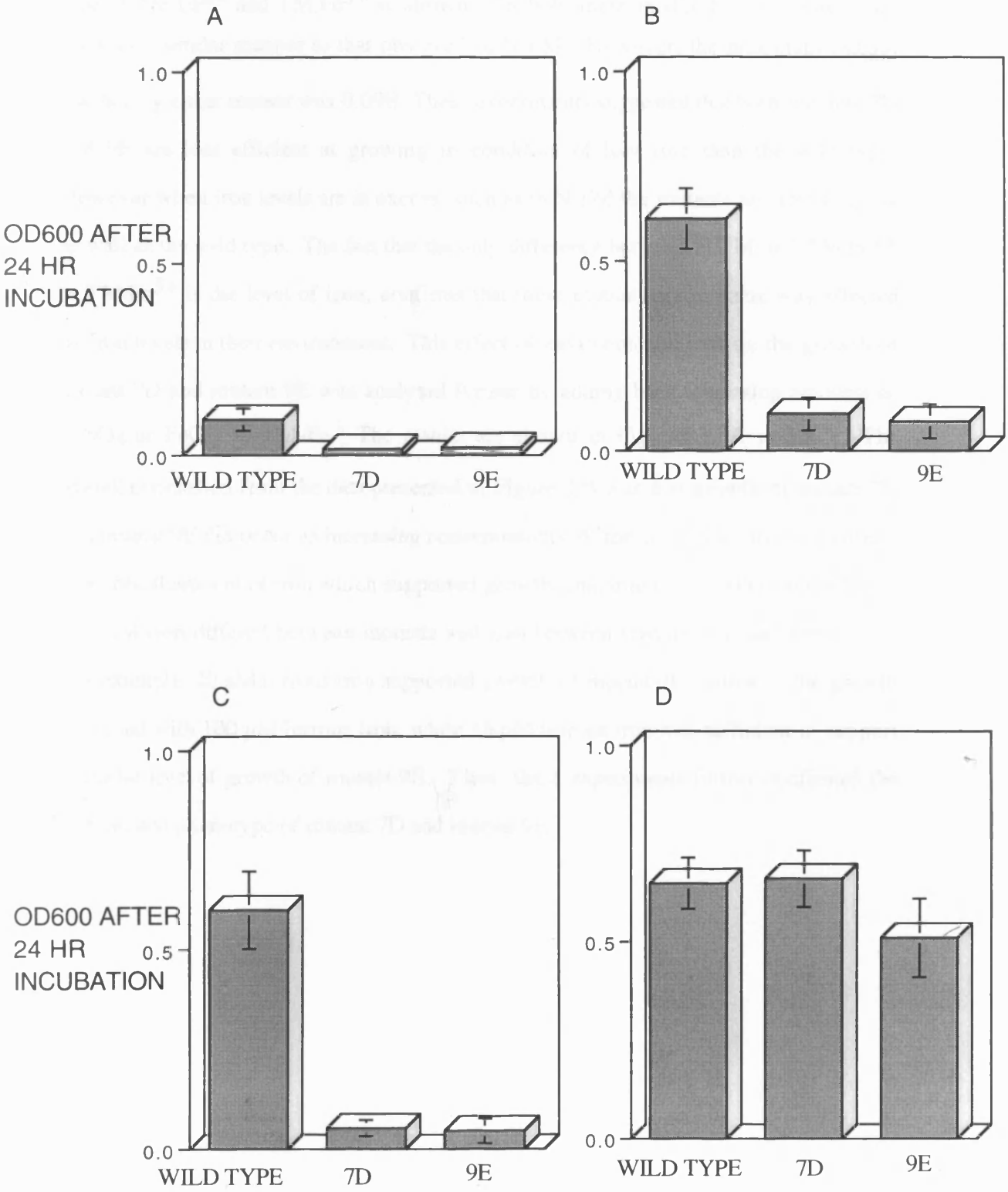
ferrous iron (0.25 μM and 0.5 μM) did support listerial growth to some extent, although it was substantially less than in the unaltered TM. Thus, the transposon library was screened for iron-related mutants using iron-depleted TM to which 1 μM $FeSO_4$ or $FeCl_3$ was added.

3.2.1 Isolation of iron-related *L. monocytogenes* mutants

The screening procedure was performed in 96-well microtiter plates containing either iron-depleted TM supplemented with 1 μM $FeSO_4$ (TM.Fe^{2+}), iron-depleted TM supplemented with 1 μM $FeNH_4$citrate (TM.Fe^{3+}) or unaltered TM (N.TM), all containing the appropriate antibiotics. *L. monocytogenes* 10403S was included in all screens, to enable comparison of the growth of the mutants with the wild type. Each mutant was inoculated from a master plate into each of the three media and incubated at 37°C for 24 hr after which the growth was examined by eye and compared to the wild type. Approximately 5,500 mutants were examined in all three media and of these 44 did not grow in TM.Fe^{2+} and TM.Fe^{3+}. No mutants were identified which did not grow in TM.Fe^{2+} but grew in TM.Fe^{3+} or vice versa. It was suspected that 28 of these 44 mutants were auxotrophs as they showed no growth in N.TM, but when examined in the complex medium, TSB, they grew. Thus the remaining 16 mutants which grew as the wild type in N.TM were examined further.

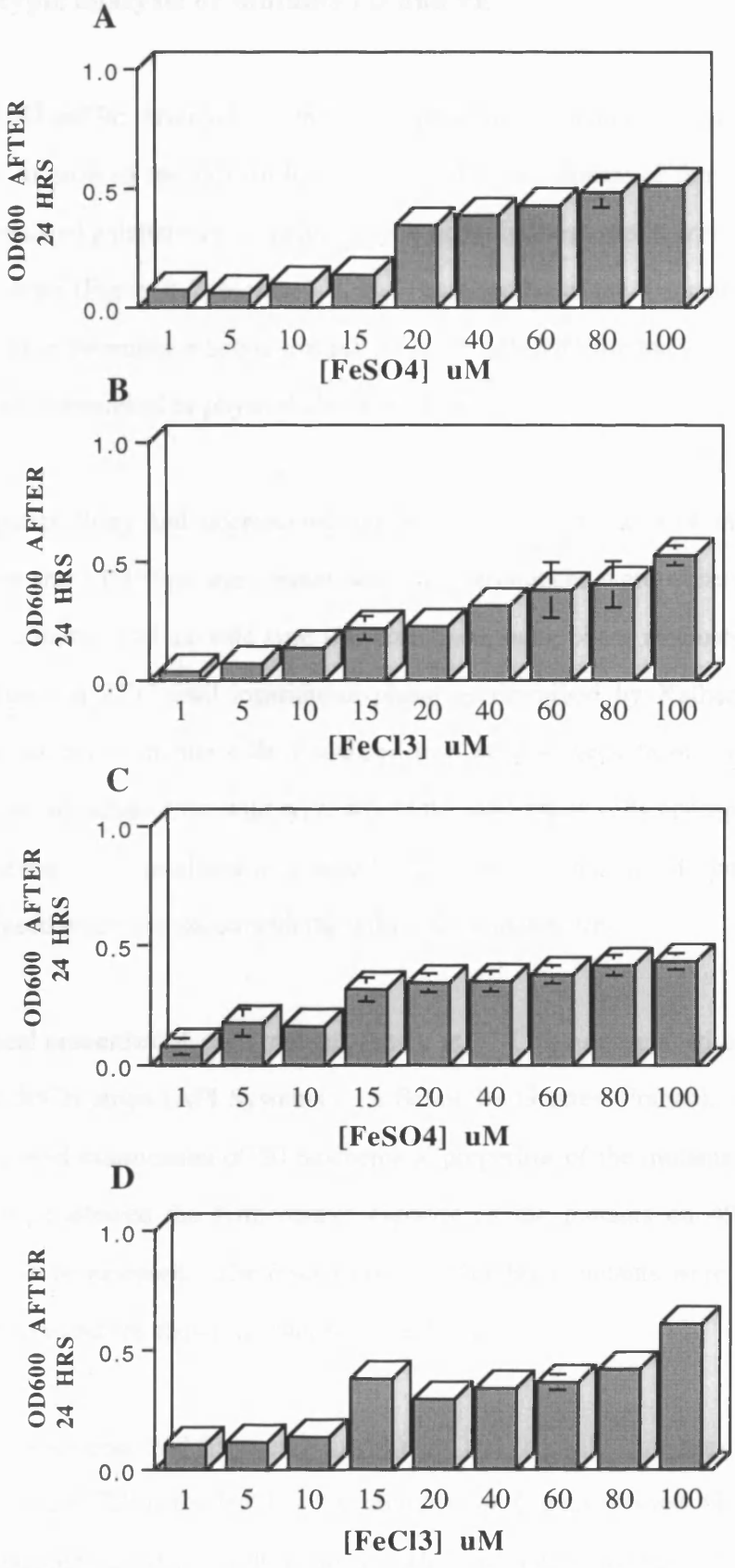
Two of these 16 mutants upon re-examination showed visibly reduced growth in both TM.Fe^{2+} and TM.Fe^{3+} when compared with the wild type strain. Both grew as the wild type in N.TM. These were designated mutants 7D and 9E. Their growth was further examined in 20 ml volumes of TM-Fe, TM.Fe^{2+}, TM.Fe^{3+} and N.TM and the results shown in Figures 3.7A-D. Figures 3.7A and 3.7D demonstrate the inhibitory effect of iron removal from N.TM. The average OD_{600} of mutants 7D, 9E and the wild type after overnight incubation in N.TM was 0.660, 0.509 and 0.648, respectively, while in TM-Fe the OD_{600} readings were 0.093, 0.01 and 0.021. This is strong

Figure 3.7. Growth of *L. monocytogenes* iron-related mutants compared with the wild type, 10403S. The OD_{600} of the wild type, and mutants 7D and 9E after 24 hr incubation at 37°C, shaking, in TM-Fe (A), TM.Fe^{2+} (B), TM.Fe^{3+} (C) and N.TM (D). These results are the mean values of eight different sets of data and the SEM is shown by the vertical bars.


A
B
C
D
OD600 AFTER
24 HR
INCUBATION
1.0
0.5
0.0
WILD TYPE
7D
9E

evidence that iron is important for the growth of *L. monocytogenes*. In Figures 3.7C and 3.7D the OD_{600}, after overnight incubation, of the mutants compared with the wild type in TM.Fe^{2+} and TM.Fe^{3+} is shown. In both these media the wild type strain grew in a similar manner to that observed in N.TM. However, the maximum OD_{600} reached by either mutant was 0.098. These experiments suggested that both mutants 7D and 9E are less efficient at growing in condition of low iron than the wild type. However when iron levels are in excess, such as in N.TM the mutants are able to grow as well as the wild type. The fact that the only difference between N.TM and TM.Fe^{2+} or TM.Fe^{3+} is the level of iron, confirms that these mutants are in some way effected by iron levels in their environment. This effect of environmental iron on the growth of mutant 7D and mutant 9E was analysed further by adding back increasing amounts of $FeSO_4$ or $FeCl_3$ to TM-Fe. The results are shown in Figures 3.8A to 3.8D. The overall conclusion from the data presented in Figure 3.8 was that growth of mutant 7D and mutant 9E did occur as increasing concentrations of iron was added to the medium. The critical amount of iron which supported growth similar to the growth observed with 100 μM iron differed between mutants and also between ferrous iron and ferric iron. For example, 20 uM ferrous iron supported growth of mutant 7D similar to the growth observed with 100 μM ferrous iron, while 15 μM ferrous iron was sufficient to support a similar level of growth of mutant 9E. Thus, these experiments further confirmed the iron-related phenotype of mutant 7D and mutant 9E.

Figure 3.8. Effect of iron concentration on the growth of mutant 7D and mutant 9E. The effect of adding increasing concentrations of $FeSO_4$ to TM-Fe on the growth of mutant 7D (A) and mutant 9E (C) was examined. Also, the effect of adding increasing amounts of $FeCl_3$ to TM-Fe on the growth of mutant 7D (B) and mutant 9E (D) was examined. The results shown represent the mean value of 3 experiments and the SEM is shown by the vertical bars.


A
OD600 AFTER 24 HRS
1.0
0.5
0.0
1 5 10 15 20 40 60 80 100
[FeSO4] uM
B
OD600 AFTER 24 HRS
1.0
0.5
0.0
1 5 10 15 20 40 60 80 100
[FeCl3] uM
C
OD600 AFTER 24 HRS
1.0
0.5
0.0
1 5 10 15 20 40 60 80 100
[FeSO4] uM
D
OD600 AFTER 24 HRS
1.0
0.5
0.0
1 5 10 15 20 40 60 80 100
[FeCl3] uM

3.3 Phenotypic analysis of mutants 7D and 9E

Both mutants 7D and 9E differed from the wild type strain, *L. monocytogenes* 10493S, in their ability to grow in media with low levels of ferrous sulphate or ferric chloride. The mutants showed a deficiency in their ability to grow in these media compared with the wild type strain (Figure 3.7, section 3.2.1). The objective of the work described in this section was to determine whether mutant 7D or 9E differed from the wild type with respect to other biochemical or physical characteristics.

The colony morphology and microscopic appearance of Gram stains of mutant 7D, mutant 9E and the wild type were examined. No differences were observed. The motility of each mutant and the wild type was examined using phase microscopy. The cells were grown at 22°C until logarithmic phase as described by Kathariou *et al.* (1995). The number of motile cells visualised in a sample taken from a mutant 9E culture was similar to that of the wild type, where the majority of cells appeared motile. The degree of motility visualised in a sample taken from a mutant 7D culture was markedly reduced when compared with the wild type or mutant 9E.

The biochemical properties of each mutant, grown at 37°C, was examined using API-20E and API-50CH strips (API Systems - La Balme les Grottes, France). The API-20E strips allowed examination of 20 biochemical properties of the mutants while the API-50CH strips allowed the fermentative capacity of the mutants on 49 different carbohydrates to be assessed. The results obtained for both mutants were compared with the wild type and are shown in Tables 3.1 and 3.2.

The only difference observed between either mutant and the wild type was the result obtained for mutant 7D in the test for the presence of β-galactosidase (Table 3.1). Mutant 7D produced a positive result in this test while mutant 9E and the wild type gave negative results. This result was confirmed by performing β-galactosidase assays as

Table 3.1. API-20E - Biochemical analysis of mutant 7D and mutant 9E

TEST	RESULTS		
	Mutant 7D	Mutant 9E	Wild type
β-galactosidase	+	-	-
arginine dihydrolase	-	-	-
lysine decarboxylase	-	-	-
ornithine decarboxylase	-	-	-
citrate utilization	-	-	-
H_2S production	-	-	-
urease	-	-	-
tryptophan deaminase	-	-	-
indole production	-	-	-
acetoin production	+	+	+
gelatinase	-	-	-
glucose fermentation/oxidation	+	+	+
mannitol fermentation/oxidation	-	-	-
inositol fermentation/oxidation	-	-	-
sorbitol fermentation/oxidation	-	-	-
rhamnose fermentation/oxidation	+	+	+
sucrose fermentation/oxidation	-	-	-
mellobiose fermentation/oxidation	-	-	-
amygdalin fermentation/oxidation	+	+	+
arabinose fermentation/oxidation	-	-	-

+ : positive reaction in the test; -: negative reaction in the test

Table 3.2. API-50CH - Fermentative capacity of mutant 7D and mutant 9E on 49 different carbohydrates

CARBOHYDRATE	RESULTS		
	Mutant 7D	Mutant 9E	Wild type
glycerol	+	+	+
erythritol	-	-	-
D-arabinose	-	-	-
L-arabinose	-	-	-
ribose	-	-	-
D-xylose	-	-	-
L-xylose	-	-	-
adonitol	-	-	-
β-methyl-D-xyloside	-	-	-
galactose	+/-	+/-	+/-
glucose	+	+	+
fructose	+	+	+
mannose	+	+	+
sorbose	+/-	+/-	+/-
rhamnose	+	+	+
dulcitol	-	-	-
inositol	-	-	-
mannitol	-	-	-
sorbitol	-	-	-
α-methyl-D-mannoside	+	+	+
α-methyl-D-glucoside	+	+	+

+ : positive reaction in the test; -: negative reaction in the test

Table 3.2 continued. API-50CH - Fermentative capacity of mutant 7D and mutant 9E on 49 different carbohydrates

CARBOHYDRATE	RESULTS		
	Mutant 7D	Mutant 9E	Wild type
N-acetyl glucosamine	+	+	+
amygdalin	+	+	+
arbutin	+	+	+
esculin	+	+	+
salicin	+	+	+
cellobiose	+	+	+
maltose	+	+	+
lactose	+	+	+
melibiose	-	-	-
sucrose	-	-	-
trehalose	+	+	+
inulin	-	-	-
melezitose	-	-	-
raffinose	-	-	-
starch	-	-	-
glycogen	-	-	-
xylitol	+	+	+
gentiobiose	+	+	+
D-turanose	-	-	-
D-lyxose	-	-	-
D-tagatose	-	-	-
D-fucose	-	-	-

Table 3.2 continued. API-50CH - Fermentative capacity of mutant 7D and mutant 9E on 49 different carbohydrates

CARBOHYDRATE	RESULTS		
	Mutant 7D	Mutant 9E	Wild type
L-fucose	-	-	-
D-arabitol	+	+	+
L-arabitol	-	-	-
gluconate	-	-	-
2-keto gluconate	-	-	-
5-keto gluconate	-	-	-

described by Miller (1972). Cells being assayed were grown at 37°C in TSB and the assays were performed at 30°C on duplicate samples. The results are shown in Table 3.3. Mutant 7D produced 1310 Miller units (MU) of β-galactosidase activity while mutant 9E and the wild type produced no β-galactosidase activity.

The haemolytic activity of both mutants was examined because it was suggested previously (Camilli *et al.*, 1990) that the *hlyA* gene, which is responsible for the haemolytic activity, may exist on a region of the listerial chromosome where *Tn* 917 is more likely to insert. This region was referred to as a "hotspot" region (Camilli *et al.*, 1990). Both mutants and the wild type were plated onto blood agar and incubated overnight at 37°C. Mutant 9E (Figure 3.9B) produced zones of haemolysis, similar to those produced by the haemolytic wild type isolate (Figure 3.9A), while mutant 7D appeared to produce greater levels of haemolysis (Figure 3.9C) than the wild type strain. These observations suggested that both mutants were haemolytic and transposition had not occurred in the *hly* "hotspot" region. The increased level of haemolysis produced by mutant 7D was not investigated further, however, the possible reasons for the increased levels of haemolysis observed for mutant 7D are discussed in section 4.3.

The ferric reductase activity of both mutants was examined as described previously (section 3.1). The results are shown in Figure 3.10. Mutant 7D (A2), mutant 9E (A3) produced a deep red colour similar to the wild type (A1), indicative of a positive ferric reductase phenotype. Ferric reductase-negative *E. coli* is shown for comparison (B).

Finally, one-dimensional SDS-PAGE protein profiles of mutant 7D and 9E were compared with those of the wild type to observe whether the mutations resulted in an observable absence or presence of a particular protein. Crude membrane protein fractions from mutants 7D, 9E and the wild type strain were separated by electrophoresis through denaturing SDS-polyacrylamide gels and visualised after

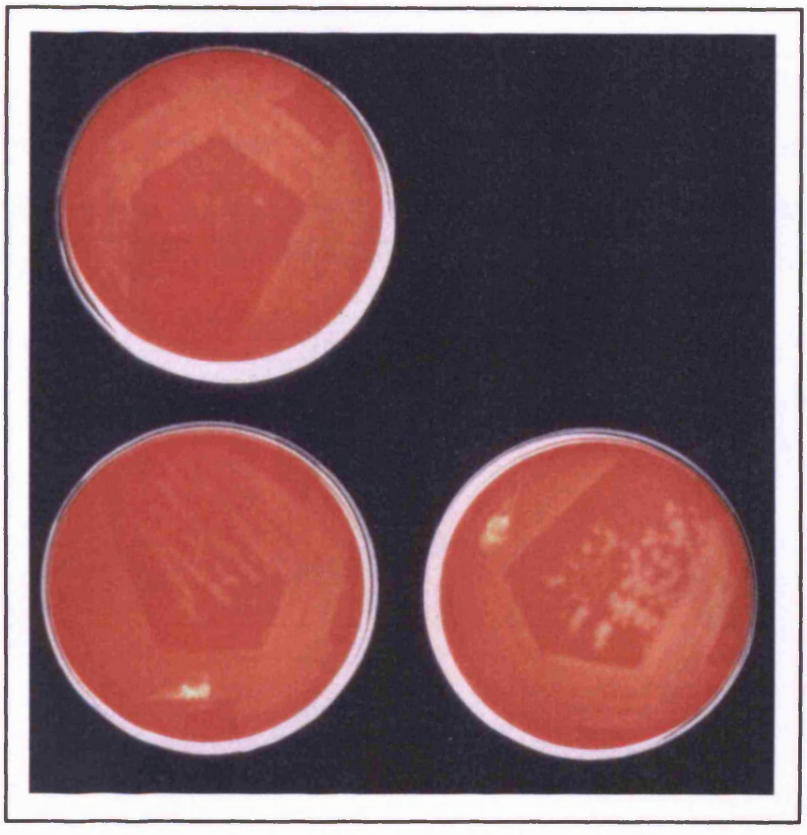
Table 3.3. β-galactosidase activity of mutant 7D and mutant 9E

ISOLATE	β-GALACTOSIDASE ACTIVITY MILLER UNITS (MU)1
Mutant 7D	1310
Mutant 9E	0
Wild type	0

[1]Standardised using the equation of Miller (1972)

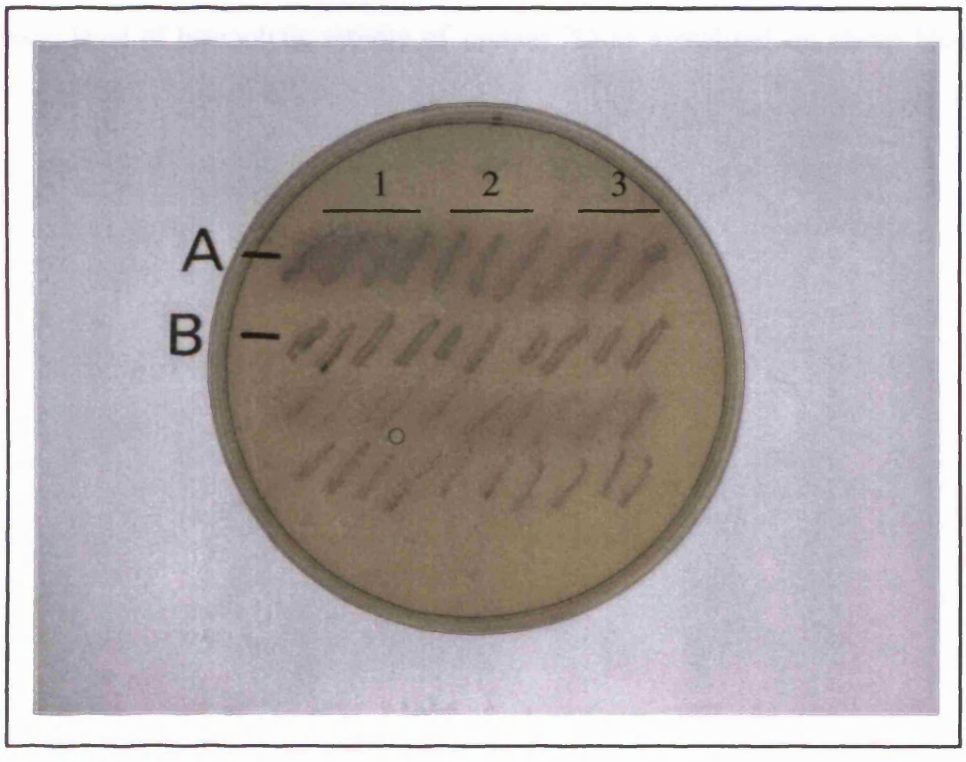
Figure 3.9. A comparison of the haemolytic activity of mutant 7D and mutant 9E with the wild type strain *L. monocytogenes* 10403S. The wild type strain (A), mutant 9E (B), and mutant 7D (C), were streaked onto TSA containing 5% sheep's blood and incubated overnight at 37°C. The haemolytic activity was observed as an area of clearing around the bacterial growth.

A



B C

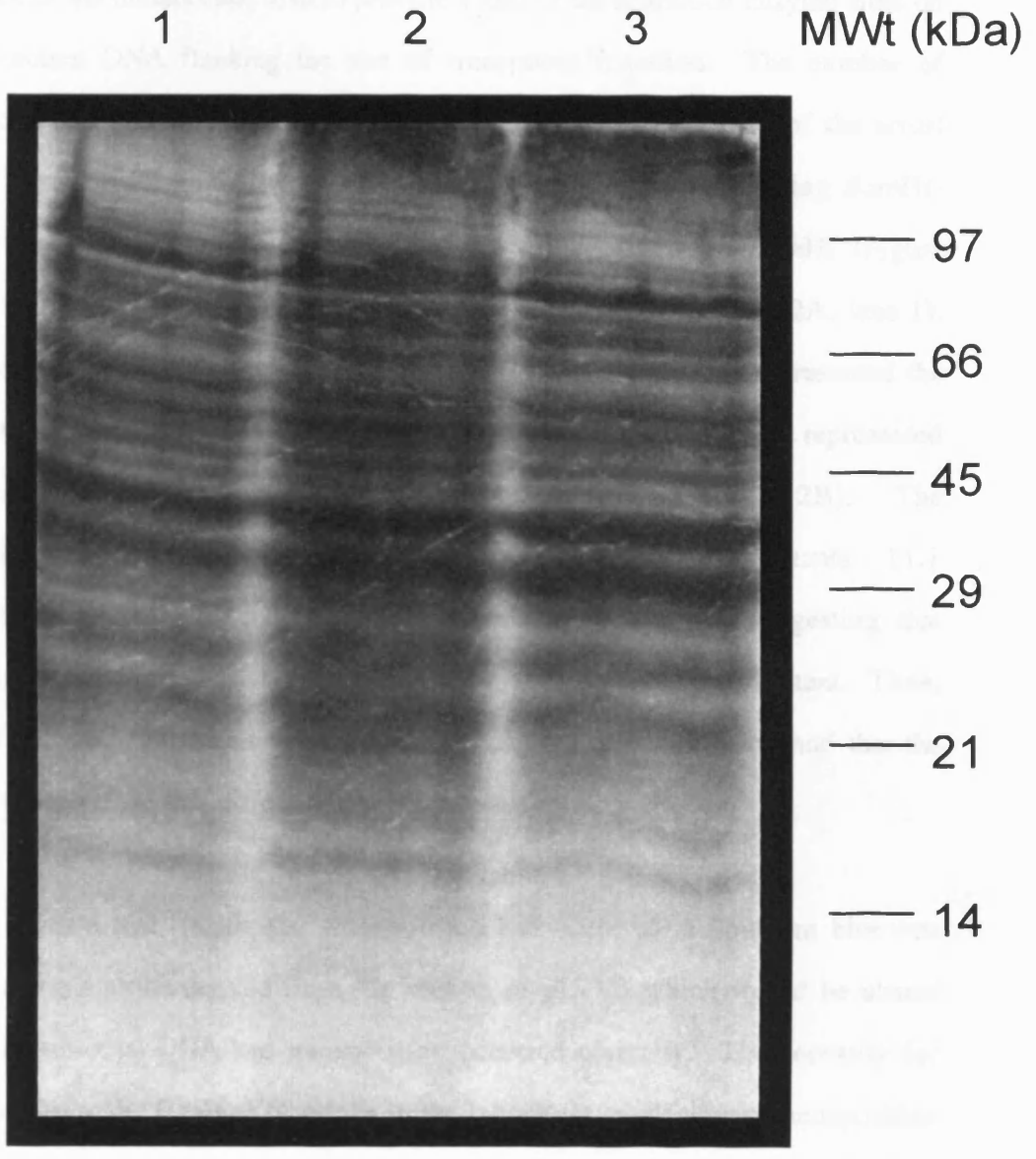
Figure 3.10. A comparison of the ferric reductase activity of mutant 7D and mutant 9E with the wild type strain *L. monocytogenes* 10403S. The ferric reductase assay of Deneer & Boychuck (1993) was used to examine both mutants and the wild type. Ferric reductase-positive isolates mutant 7D (A2), mutant 9E A3) and the wild type (A1) are represented by a red colour surrounding the streaks. The ferric reductase-negative *E. coli* Sure (B) is represented by the streaks with no red colouration.


1
2
3
A
B

Coomassie blue staining. The profiles shown in Figure 3.11 represent protein extracts from the wild type strain (Lane 1), mutant 7D (Lane 2) and mutant 9E (Lane 3). No differences between the profiles were observed.

Thus, to summarise the findings of this section, the mutants appear to be identical to the wild type strain with respect to: colony morphology, ferric reductase activity, one-dimensional SDS-PAGE protein profiles and a wide range of biochemical characteristics. The only differences observed were: (1) the reduced motility of mutant 7D compared with the wild type and mutant 9E, (2) the expression of β-galactosidase by mutant 7D compared with the lack of activity by mutant 9E and the wild type and (3) the increased level of haemolytic activity of mutant 7D as visualised on sheep blood agar.

Figure 3.11. Comparison of SDS-PAGE protein profiles from mutant 7D, mutant 9E and the wild type strain *L. monocytogenes* 10403S. Coomassie blue-stained SDS-PAGE crude membrane profiles of *L. monocytogenes* 10403S (Lane 1), mutant 7D (lane 2), mutant 9E (lane 3). Approximate molecular weights are shown on the right.


1
2
3
MWt (kDa)
97
66
45
29
21
14

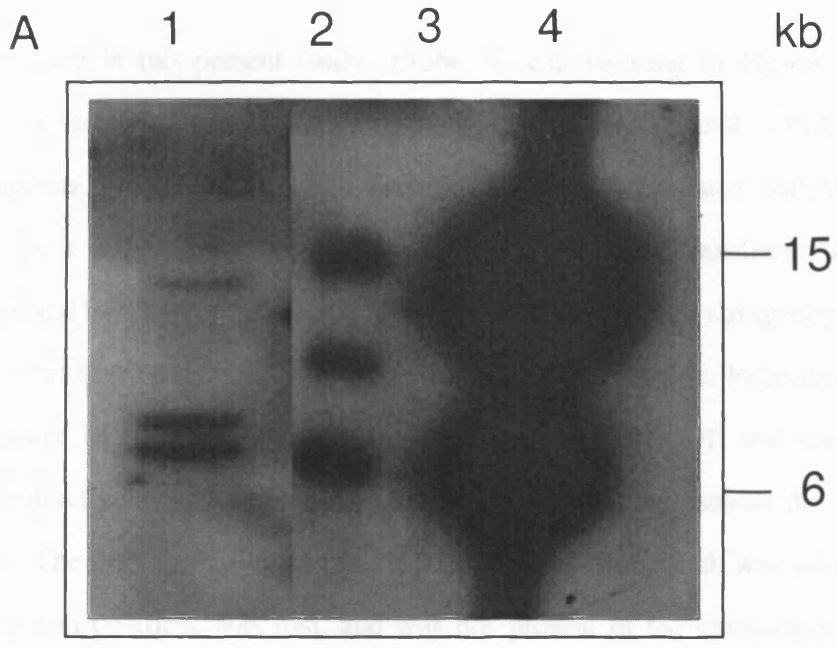
3.4 Southern blot analysis of mutants 7D and 9E and restriction mapping the flanking chromosomal DNA

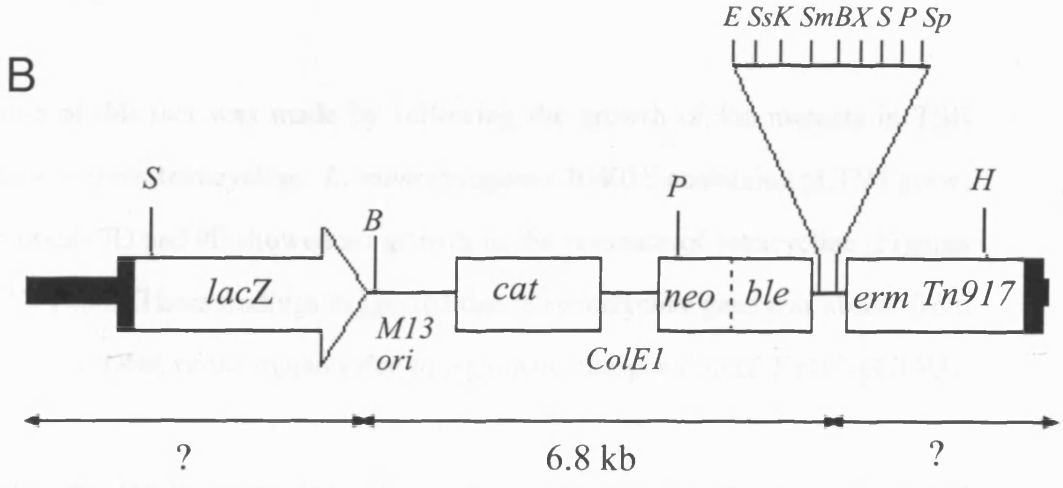
Having isolated two mutants, 7D and 9E, with defective growth in low iron media, Southern blots were performed to confirm the presence of the transposon in the chromosome of the mutants and also to provide a map of the restriction enzyme sites on the chromosomal DNA flanking the site of transposon insertion. The number of transposition events that had occurred in each mutant and a comparison of the actual location of transposition in the two mutants was investigated by hybridising *Bam*HI-digested DNA from mutant 7D and 9E with pLTV3 digested with *Bam*HI (Figure 3.12A). For mutant 7D (Figure 3.12A, lane 2) and mutant 9E (Figure 3.12A, lane 1), 3 bands were observed. The 6.8 kb band, common to both mutants represented the internal *Bam*HI fragment of the transposon, while the remaining two bands represented the flanking DNA at either side of the internal fragment (Figure 3.12B). The approximate sizes of these flanking bands were different between the mutants - 11.1 and 19.1 kb in mutant 7D and 7.7 and 16.2 kb in mutant 9E, suggesting that transposition had occurred in different sites of the chromosome of each mutant. Thus, it appeared that both mutations were a result of one transposition event and that the location of the insertion was different in both mutants.

To further confirm that "legitimate" transposition had occurred, a Southern blot was performed using a probe derived from the section of pLTV3 which would be absent from the chromosomal DNA had transposition occurred correctly. The necessity for this blot was due to the findings of others in the laboratory of illegitimate transposition of *Tn*917-pLTV3 into the listerial chromosome (A. Passos, personal communication; Alexander, 1994). These workers demonstrated the occurrence of illegitimate transposition and transposon rearrangements by Southern blot analysis. Using a probe derived from the section of pLTV3 which would be lost during normal transposition, both these workers observed hybridisation to chromosomal DNA from their mutants

Figure 3.12A. Southern hybridisation of chromosomal DNA from mutant 7D and 9E using pLTV3 as a probe. *Bam*HI-digested chromosomal DNA from *L. monocytogenes* 7D (lane 2), 9E (lane 1), wild type (lane 3) and plasmid pLTV3 (lane 4) were analysed on a 0.6% (w/v) agarose gel and transferred to a nylon membrane. The whole of pLTV3 digested with *Bam*HI was labelled with [α-^{32}P] dCTP and used for hybridising under conditions of high stringency. The membrane was washed 3 times in 0.1X SSC, 0.1% (w/v) SDS at 65°C for 20 min. Approximate fragment sizes are indicated on the right.

Figure 3.12B. Hypothetical diagram of transposition in mutant 7D or 9E. The presence of *Tn*917-pLTV3 in the chromosome of either mutant 7D or 9E is shown diagrammatically. The 3 hybridising bands observed in the Southern blot above (Figure 3.12A, lanes 1 and 2) are indicated in the diagram - the internal 6.8 kb fragment of the transposon and the 2 flanking fragments. The exact size of flanking fragments is unknown and the diagram is not to scale. Features of the transposon include the promoterless copy of the *lacZ* gene, the M13 and ColE1 origins of replication, the antibiotic resistant markers, *neo*, *ble* and *erm* and the polylinker. Restriction sites are as as follows: *B*, *Bam*HI; *E*, *Eco*RI; *H*, *Hind*III; *K*, *Kpn*I; *P*, *Pst*I; *S*, *Sal*I; *Sm*, *Sma*I; *Sp*, *Sph*I; *Ss*, *Sst*I; *X*, *Xba*I.


A
1
2
3
4
kb
15
6


B
E SsK SmBX S P Sp
S
B
P
H
lacZ
cat
neo
ble
erm Tn917
M13
ori
ColE1
?
6.8 kb
?

and thus, concluded that some type of transposon rearrangement had taken place and resulted in the presence of the unexpected region of pLTV3 in the chromosome of the mutant (A. L. Passos, personal communication).

The probe used in this present study, Probe A, can be seen in Figure 3.13A and includes the tetracycline resistance gene, *tet*. Chromosomal DNA from *L. monocytogenes* 7D, 9E, 10403S and plasmid pLTV3 was digested with *Bam*HI and separated on a 0.6% (w/v) agarose gel. The DNA was transferred to a nylon membrane, and hybridised with probe A under conditions of high stringency. The three resulting hybridisation patterns are shown in Figure 3.13B. No hybridisation bands were observed in lanes 1, 2 or 3, representing mutant 7D, 9E and the wild type 10403S, respectively. A band was observed in lane 4 and represented the 15 kb band of pLTV3. The findings indicated that the region of pLTV3 which was expected to be lost during transposition, was lost, and was not present in the chromosome of either mutant to hybridise with Probe A. This suggested that, in contrast to the previous findings of A. L. Passos and J Alexander of illegitimate transposition events, transposition in both mutant 7D and mutant 9E of *Tn*917-pLTV3 appeared to have occurred correctly.

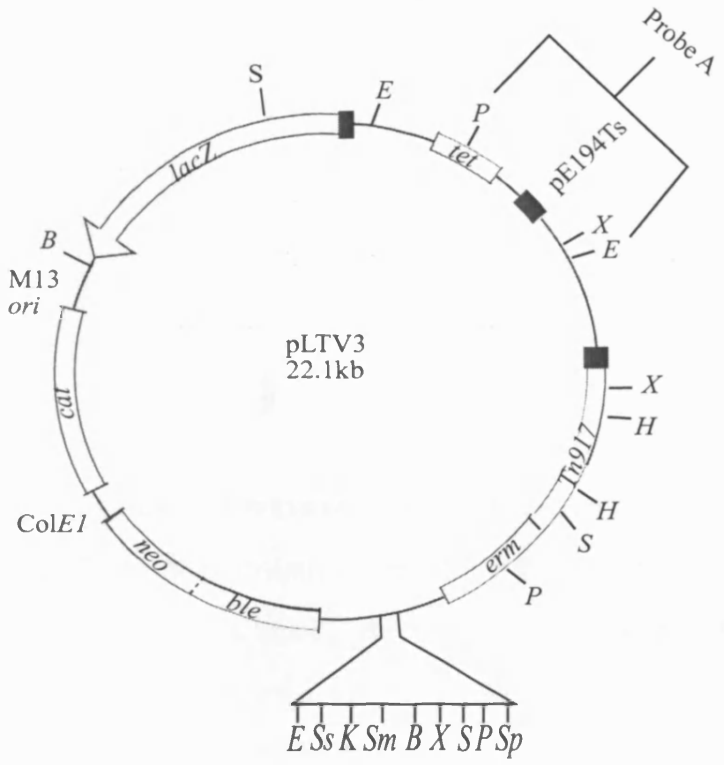
Confirmation of this fact was made by following the growth of the mutants in TSB either with or without tetracycline. *L. monocytogenes* 10403S containing pLTV3 grew, whereas mutants 7D and 9E showed no growth in the presence of tetracycline (Figures 3.14A and 3.14B). These findings suggested that the tetracycline gene was absent from the chromosomal DNA of the mutants due to legitimate transposition of *Tn*917-pLTV3.

At this stage the results suggested that: firstly, only one transposition event had occurred in each mutant; secondly, this event appeared to involve "normal" transposition as witnessed by the loss of the tetracycline resistance gene; and thirdly the location of the transposon in each mutant was different. Southern blots were then

Figure 3.13A. Map of pLTV3 showing Probe A. Probe A was used in the Southern blot shown in Figure 3.13B below and represents the 2.8 kb *Pst*I - *Eco*RI fragment specific to pLTV3, containing the tetracycline resistance gene, *tet.* Restriction sites are as as follows: *B, Bam*HI; *E, Eco*RI; *H, Hind*III; *K, Kpn*I; *P, Pst*I; *S, Sal*I; *Sm, Sma*I; *Sp, Sph*I; *Ss, Sst*I; *X, Xba*I.

Figure 3.13B. Southern hybridisation of chromosomal DNA from mutant 7D and 9E using Probe A. *Bam*HI-digested chromosomal DNA from mutant 7D (lane 1), mutant 9E (lane 2), wild type (lane 3) and plasmid pLTV3 (lane 4) were analysed on a 0.6% (w/v) agarose gel and transferred to a nylon membrane. Probe A (shown in Figure 3.13A) was labelled with [α-^{32}P] dCTP and used for hybridising under conditions of high stringency. The membrane was washed 3 times in 0.1X SSC, 0.1% (w/v) SDS at 65°C for 20 min. Approximate fragment sizes are indicated on the right.

A


Probe A
S
E
P
lacZ
tet
pE194Ts
X
E
B
M13
ori
pLTV3
22.1kb
X
H
cat
Tn917
H
S
ColE1
neo
ble
erm
P
E Ss K Sm B X S P Sp

B

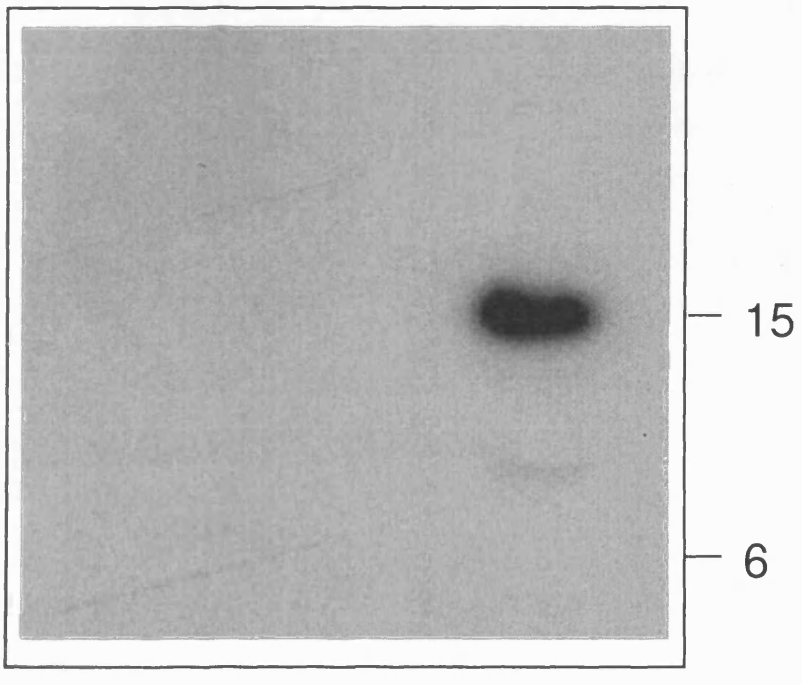
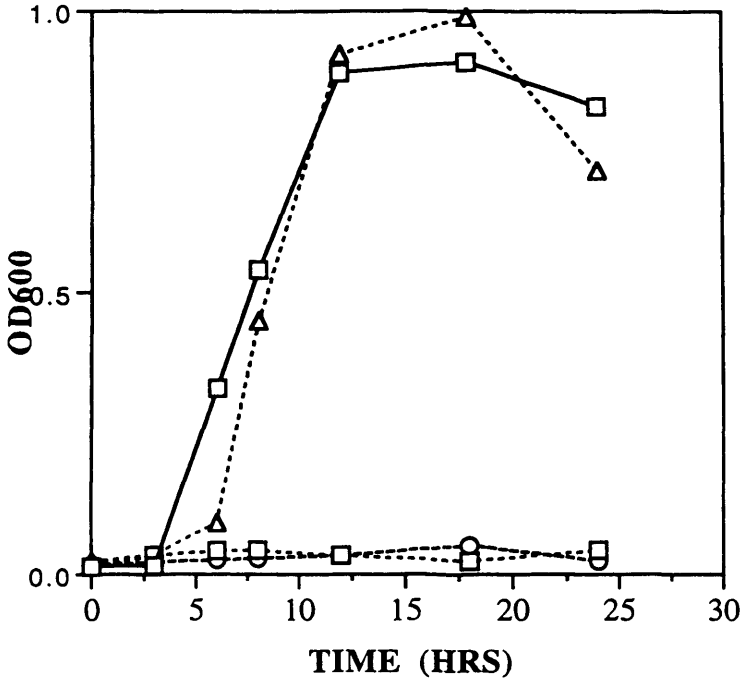
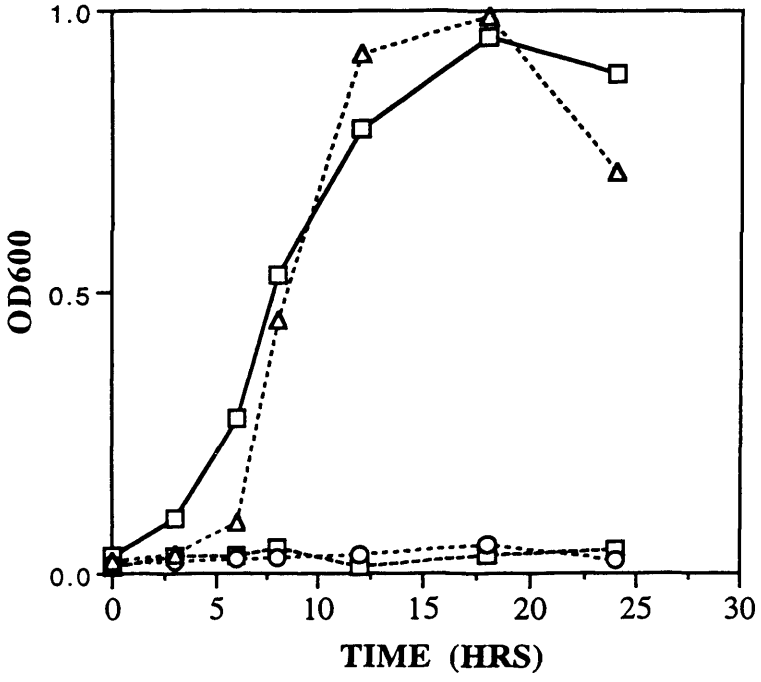

1
2
3
4
kb
15
6

Figure 3.14. The effect of tetracycline on the growth of *L. monocytogenes* 7D and 9E. *L. monocytogenes* mutants 7D (Figure 3.14A) and 9E (Figure 3.14B) were grown in TSB containing erythromycin (10 μg/ml) and lincomycin (25 μg/ml) either with (-----) or without(_____) tetracycline (20 μg/ml). Cells were incubated at 37°C, shaking for 24 hr and the OD_{600} recorded at regular intervals. *L. monocytogenes* 10403S containing pLTV3 was used as the positive control (--Δ--) while *L. monocytogenes* 10403S alone (--o--) was used as the negative control.

A


1.0
0.5
0.0
OD600
0
5
10
15
20
25
30
TIME (HRS)

B


1.0
0.5
0.0
OD600
0
5
10
15
20
25
30
TIME (HRS)

performed which would enable the construction, for each mutant, of a partial restriction map of the chromosomal DNA flanking the *erm*-proximal end of the transposon.

Two different probes, *Bam*HI-digested pLTV3 or, Probe B, a *Sal*I-*Eco*RI fragment of pLTV3 were used to hybridise chromosomal DNA from both mutants. These probes can be seen in Figure 3.15. Chromosomal DNA from mutants 7D and 9E digested with *Hind*III (lanes 1), *Kpn*I (lanes 2), *Pst*I (lanes 3), *Sma*I (lanes 4), *Sph*I (lanes 5), *Xba*I (lanes 6), *Xho*I (lanes 7) was hybridised with *Bam*HI-digested pLTV3 (Figures 3.16A and 3.17B) or with Probe B (Figures 3.16B and 3.17B). In each blot, *Bam*HI-restricted wild type DNA and pLTV3 were included as negative and positive controls (Figure 3.16 A, B and 3.17A, B, lanes 9 and 10, respectively). These blots enabled the determination of which restriction enzyme sites were present in the flanking DNA, at the *erm*-proximal side of transposition. Mapping the DNA flanking the *erm*-proximal side of the transposon was necessary, as this was the side which was to be rescued at a later stage of this investigation (section 3.5).

As this part of the investigation was aimed at constructing restriction maps of the *erm*-proximal end of transposition in both mutants, the band which represented the *erm*-proximal flanking DNA was identified by hybridisation with Probe B (Figures 3.16A and 3.17A, bands indicated by the arrows). This probe would only hybridise with DNA from the *erm*-proximal side of the transposon. On the blots using *Bam*HI-digested pLTV3 as a probe (Figures 3.16A and 3.17A), the bands which represent the *erm*-proximal flanking DNA are indicated by the arrows and the other bands which can be seen in Figures 3.16A and 3.17A, represent the hybridisation which can occur with the whole vector as a probe to DNA. These bands represent bands which are internal to *Tn*917-pLTV3 or to DNA flanking the *erm*-distal side of transposition. For construction of restriction maps of the DNA flanking the *erm*-proximal side of the transposon, these bands were not of importance and thus were not analysed further.

Figure 3.15. Map of pLTV3 showing Probe B. Probe B was used in the Southern blots shown in Figure 3.16B and 3.17B . Probe B is a 1.4 kb *Sal*I - *Eco*RI fragment specific to pLTV3. Restriction sites are as as follows: *B*, *Bam*HI; *E*, *Eco*RI; *H*, *Hind*III; *K*, *Kpn*I; *P*, *Pst*I; *S*, *Sal*I; *Sm*, *Sma*I; *Sp*, *Sph*I; *Ss*, *Sst*I; *X*, *Xba*I.

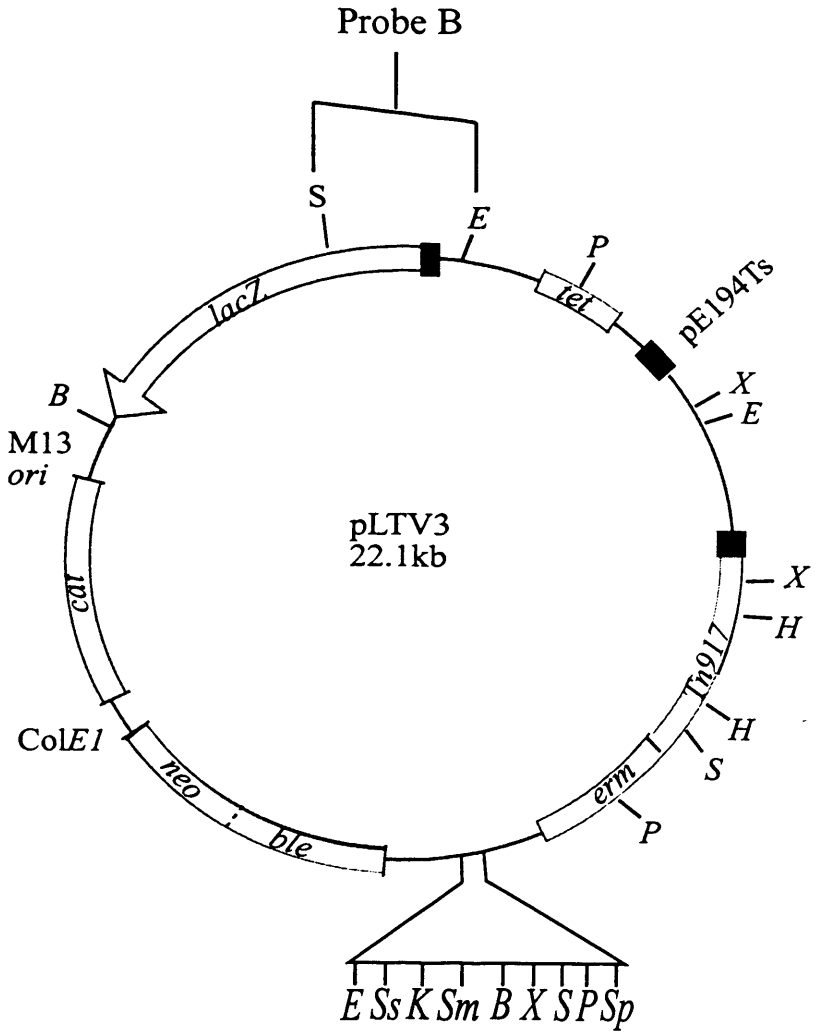
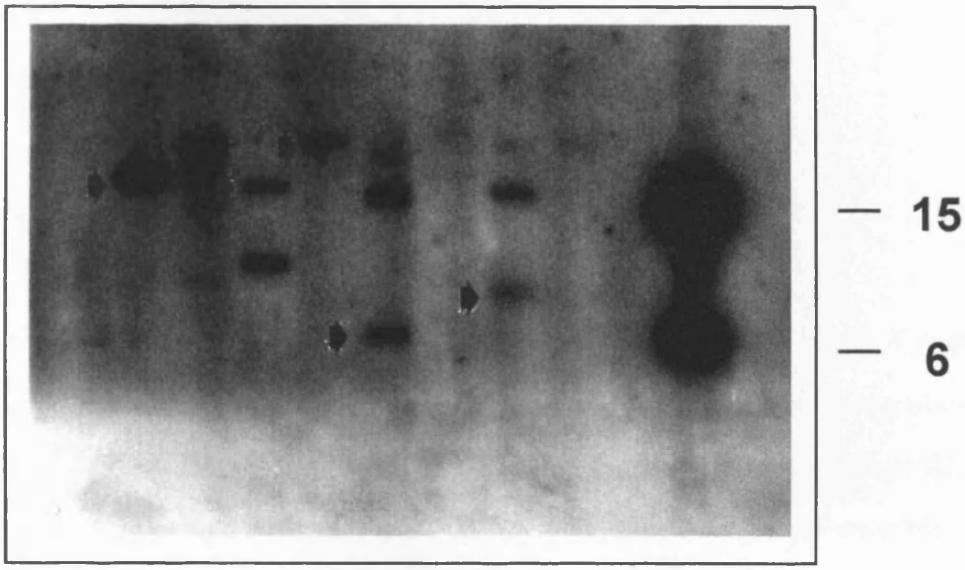

Probe B
S
E
P
tet
pE194Ts
X
E
X
H
H
S
P
Tn917
erm
lacZ
B
M13
ori
cat
ColE1
neo
ble
pLTV3
22.1kb
E Ss K Sm B X S P Sp

Figure 3.16. Southern hybridisation of chromosomal DNA from mutant 7D using pLTV3 as a probe (Figure 3.16A) or Probe B (Figure 3.16B). *Hind*III (lane 1), *Kpn*I (lane 2), *Pst*I (lane 3), *Sma*I (lane 4), *Sph*I (lane 5), *Xba*I (lane 6), *Xho*I (lane 7) - digested chromosomal DNA were analysed on a 0.6% (w/v) agarose gel and transferred to a nylon membrane. Lane 8 represent chromosomal DNA from mutant 9E digested with *Hind*III, lane 9 represent wild type DNA digested with *Bam*HI and lane 10 represent pLTV3 digested with *Bam*HI. Approximate fragment sizes are indicated on the right. The probes were labelled with [α-^{32}P] dCTP and used for hybridising under conditions of high stringency. The membranes were washed 3 times in 0.1X SSC, 0.1% (w/v) SDS at 65°C for 20 min.

A



B

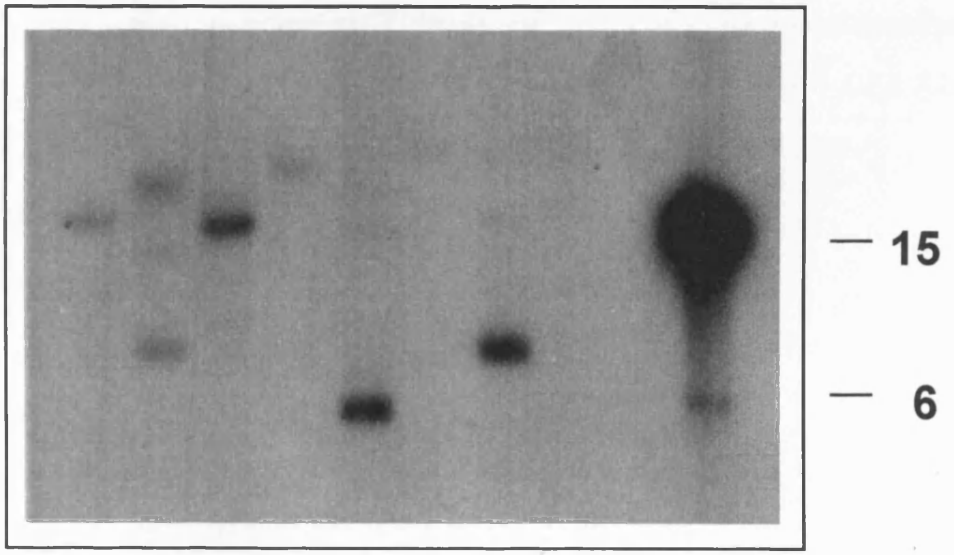
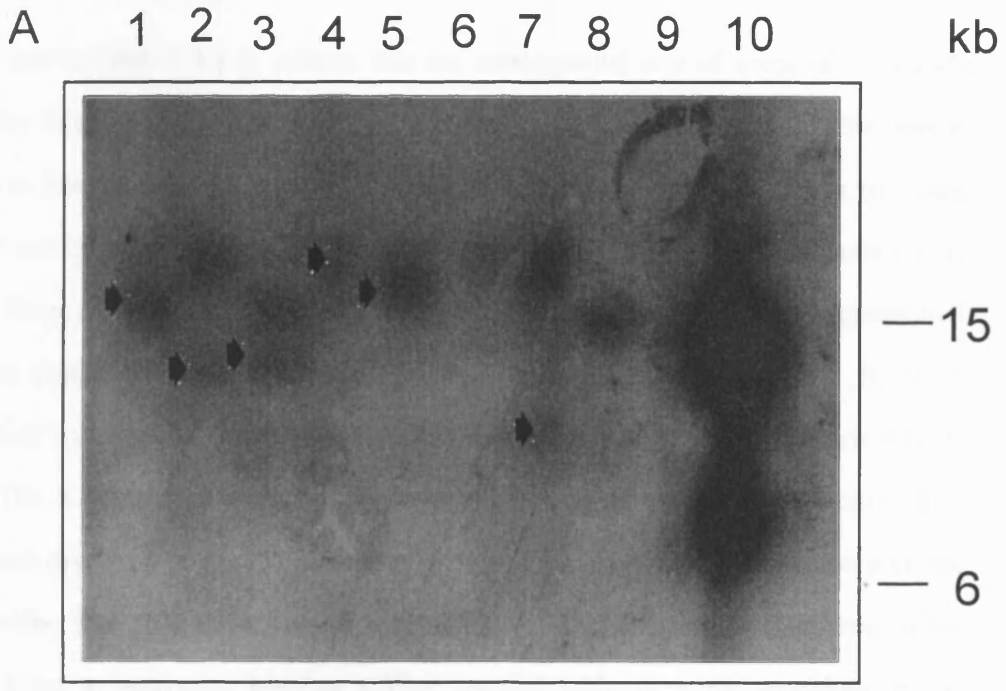
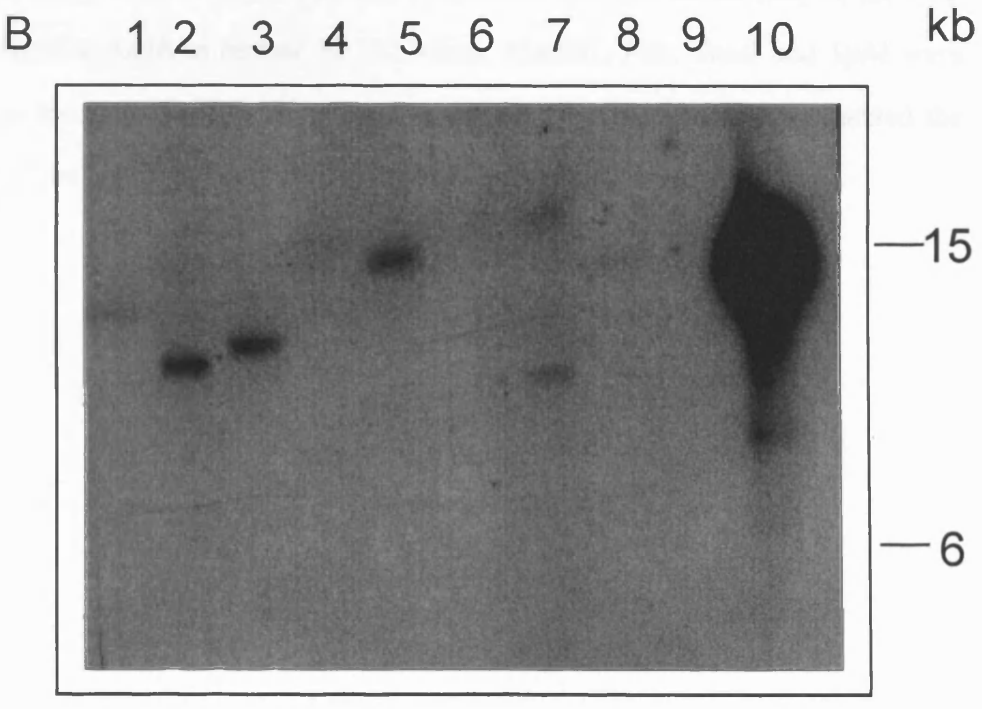


Figure 3.17. Southern hybridisation of chromosomal DNA from mutant 9E using pLTV3 as a probe (Figure 3.17A) or Probe B (Figure 3.17B). *Hind*III (lane 1), *Kpn*I (lane 2), *Pst*I (lane 3), *Sma*I (lane 4), *Sph*I (lane 5), *Xba*I (lane 6), *Xho*I (lane 7) - digested chromosomal DNA were analysed on a 0.6% (w/v) agarose gel and transferred to a nylon membrane. Lane 8 represent chromosomal DNA from mutant 7D digested with *Hind*III, lane 9 represent wild type DNA digested with *Bam*HI and lane 10 represent pLTV3 digested with *Bam*HI. Approximate fragment sizes are indicated on the right. The probes were labelled with [α-^{32}P] dCTP and used for hybridising under conditions of high stringency. The membranes were washed 3 times in 0.1X SSC, 0.1% (w/v) SDS at 65°C for 20 min.


A
1 2 3 4 5 6 7 8 9 10
kb
15
6


B
1 2 3 4 5 6 7 8 9 10
kb
15
6

The expected sizes of the *erm*-proximal flanking DNA bands, and the approximate sizes of the bands obtained are listed in Table 3.4.

From the data in Table 3.4 it is evident that the experimental size of some of the bands was smaller that the size expected as calculated using the map of pLTV3. The reason for this was not known and was not investigated further. However, these enzymes sites were not included in the final restriction maps of the DNA of the mutants (*Sph*I and *Xho*I for mutant 7D and *Xho*I and *Kpn*I for mutant 9E). Also, when chromosomal DNA from mutant 7D was digested with *Kpn*I and hybridised with Probe B, three hybridisation bands were observed when only one band was expected (Figure 3.16B, lane 2). The reason for this observation was not known, although, it may have been due to partial digestion of the chromosomal DNA. Consequently, a *Kpn*I site was not included in the final restriction map of mutant 7D. It was also impossible, from these Southern blots, to determine whether a *Xba*I restriction site occurred anywhere in the flanking DNA of mutant 7D or mutant 9E as no bands were observed (Figures 3.16A, 3,16B, 3.17A, 3.17B, lane 6). However, at this stage of the investigation, enough data were produced to allow the construction of restriction maps of the chromosomal DNA flanking the *erm*-proximal side of insertion in both mutants. These are shown in Figure 3.18. Restriction sites, *Hind*III, *Pst*I and *Sma*I were included on the map of the *erm*-proximal flanking DNA in mutant 7D (A) while, *Hind*III, *Pst*I, *Sma*I and *Sph*I were included on the map of the flanking DNA in mutant 9E (B). These maps enabled the next stage of the investigation to be undertaken (section 3.5).

Table 3.4. DNA bands expected and obtained, from the *erm*-proximal flanking DNA as determined by Southern analysis; hybridising chromosomal DNA from mutant 7D or 9E with probes, *Bam*HI-digested pLTV3 or Probe B.

Enzyme used for DNA digest	Band expected[1](kb)	Band obtained[2](kb)	
		Mutant 7D	Mutant 9E
*Hind*III	>12.2[3]	18.4	12.9
*Kpn*I	>9.7[3]	10.1[4]	9.1
*Pst*I	>8.0[3]	13.6	10.5
*Sma*I	>9.1[3]	19.4	17.4
*Sph*I	>8.7[3]	5.8	15.7
*Xba*I	>9.6[3]	None	None
*Xho*I	>9.6[3]	8.1	9.0

[1]Sizes calculated using the map of pLTV3

[2]Sizes calculated from the Southern blots in Figures 3.16 and 3.17

[3]Approximate sizes in kb

[4]Two other bands also present - see below (section 3.4) for discussion of these

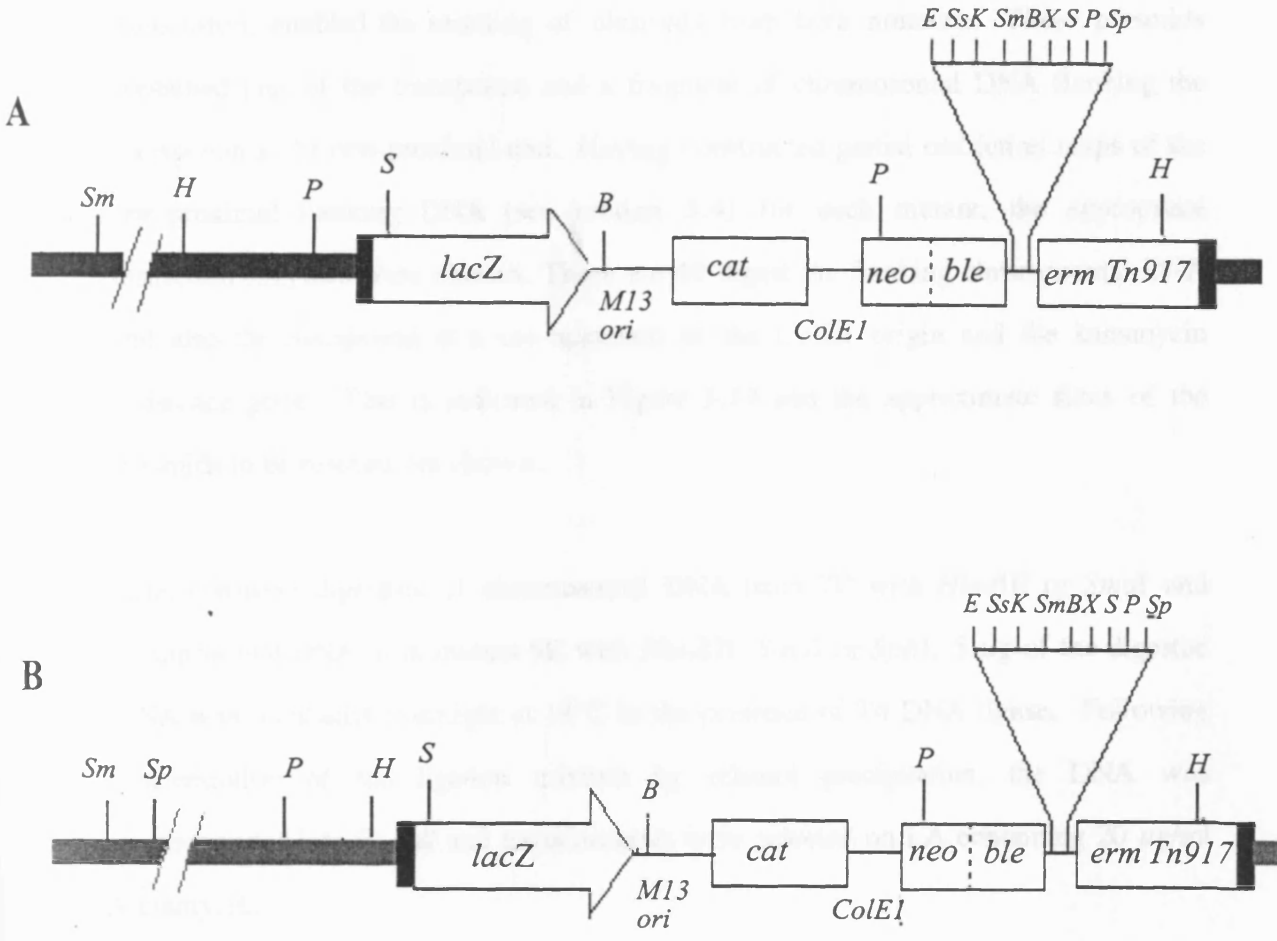

A
E SsK SmBX S P Sp
Sm
H
P
S
B
P
H
lacZ
cat
neo
ble
erm Tn917
M13
ori
ColE1
B
E SsK SmBX S P Sp
Sm
Sp
P
H
S
B
P
H
lacZ
cat
neo
ble
erm Tn917
M13
ori
ColE1

Figure 3.18. Partial restriction enzyme maps of flanking chromosomal DNA from mutant 7D (Figure 3.18A) and mutant 9E (Figure 3.18B). Diagram showing Tn917-pLTV3 transposition in the chromosome of mutant 7D (A) and mutant 9E (B). The thick solid lines in each diagram represent the flanking chromosomal DNA. Restriction sites are as follows: *B, BamHI; E, EcoRI; H, HindIII; K, KpnI; P, PstI; S, SalI; Sm, SmaI; Sp, SphI; X, XbaI.* The other features of the transposon were described previously in Figure 3.12B .

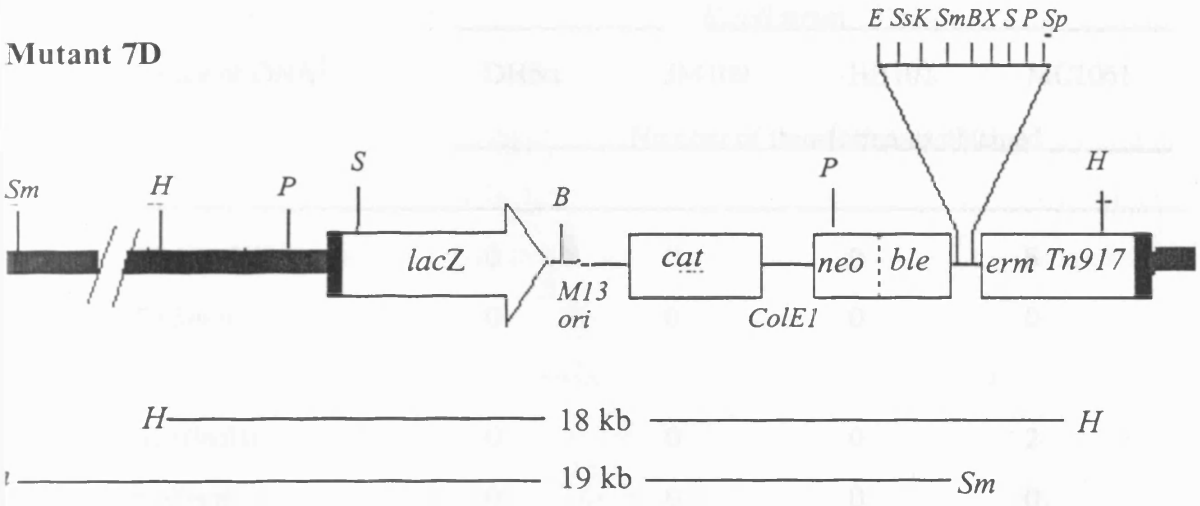
3.5 Rescuing flanking chromosomal DNA from mutants 7D and 9E and subcloning into pBluescript^{SK} for sequencing

The presence of the ColE1 origin of replication within the transposon, in addition to the kanamycin resistance gene (*neo*) and an appropriate restriction enzyme site on the transposon, enabled the rescuing of plasmids from both mutants. These plasmids contained part of the transposon and a fragment of chromosomal DNA flanking the transposon at the *erm*-proximal end. Having constructed partial restriction maps of the *erm*-proximal flanking DNA (see section 3.4) for each mutant, the appropriate restriction enzymes were chosen. These would digest the flanking chromosomal DNA and also the transposon at a site upstream of the ColE1 origin and the kanamycin resistance gene. This is indicated in Figure 3.19 and the approximate sizes of the plasmids to be rescued are shown.

After complete digestion of chromosomal DNA from 7D with *Hind*III or *Sma*I and chromosomal DNA from mutant 9E with *Hind*III, *Sma*I or *Sph*I, 5 μg of the digested DNA was incubated overnight at 14°C in the presence of T4 DNA ligase. Following concentration of the ligation mixture by ethanol precipitation, the DNA was electroporated into *E. coli* and transformants were selected on LA containing 20 μg/ml kanamycin.

Four different *E. coli* strains were used in these attempts to rescue the various plasmids from either mutant. These were DH5α, JM109, HB101, MC1061. Initially, the plasmid rescue experiments were performed in DH5α or JM109. After repeated attempts using DH5α and JM109, no kanamycin resistant transformants containing the self-ligated plasmids were obtained (Table 3.5). In retrospect, this result was not surprising as it has been reported recently (Guiterrez *et al.*, 1996) that when rescuing *erm*-containing DNA in *E. coli*, a strain with a *rpsl*20 mutation should be used as this enables the strain to overcome the problem of toxicity which may occur due to the

Mutant 7D



Mutant 9E

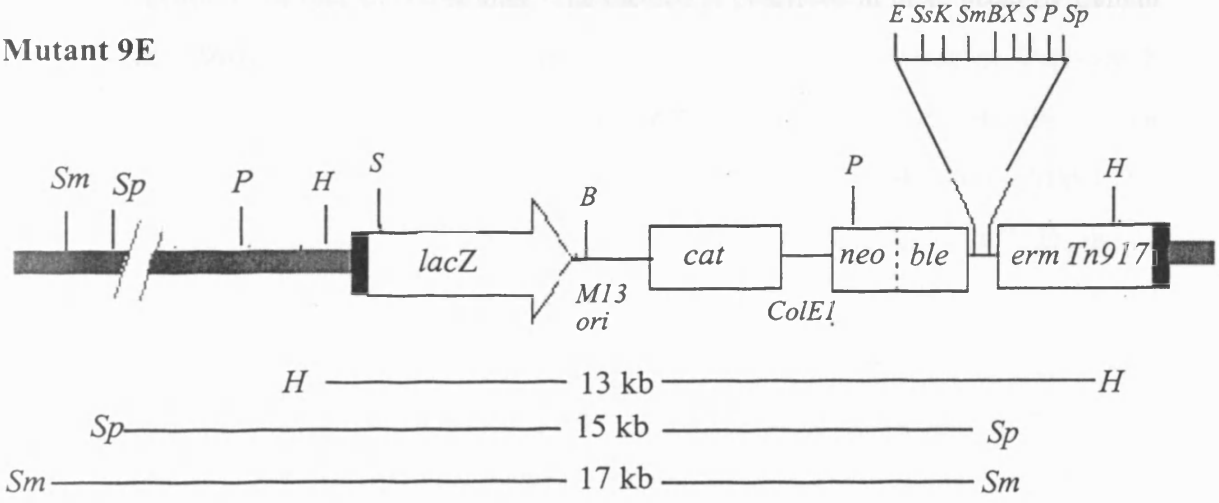


Figure 3.19. Diagrammatic representation of transposition in mutants 7D and 9E indicating the plasmids to be rescued. Diagram showing the occurrence of *Tn*917-pLTV3 in the chromosome of mutant 7D and mutant 9E (more detail of the diagram given in Figure 3.18). The restriction enzymes used to digest the chromosomal DNA prior to self ligation and electroporation into *E.coli* MC1061 are indicated in bold. The approximate sizes of the different plasmids which were to be rescued are shown below the diagram.

Table 3.5. *E.coli* transformants which contained rescued plasmids from mutant 7D or 9E: the use of different bacterial strains for transformation.

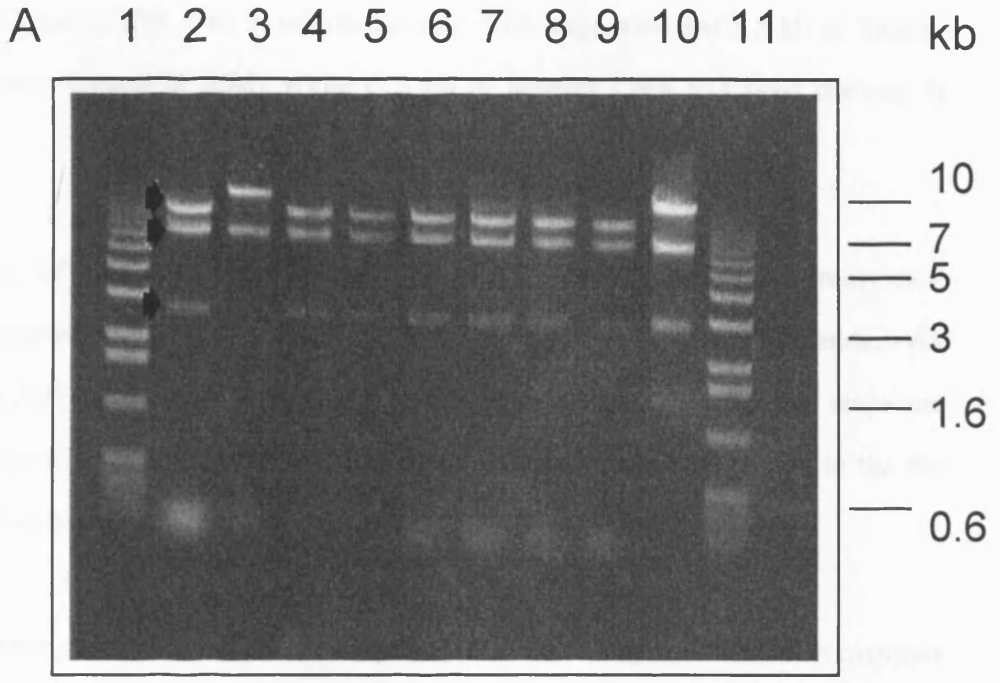
Source of DNA[1]	*E.coli* strain			
	DH5α	JM109	HB101	MC1061
	Number of transformants obtained			
7D-*Hind*III	0	0	0	8
7D-*Sma*I	0	0	0	0
9E-*Hind*III	0	0	0	2
9E-*Sma*I	0	0	0	0
9E-*Sph*I	0	0	0	0

[1]5 μg of chromosomal DNA from either mutant was digested with one of the stated restriction enzymes and self-ligated in a final volume of 150 μl prior to electroporation into each of the four *E. coli* strains. The method is described in more detail by Camilli *et al.* (1990).

presence of the *erm* gene product (Youngman, 1990; Guiterrez *et al.*, 1996). As a consequence, HB101 and MC1061 were used.

As shown in Table 3.5, 8 kanamycin resistant transformants were obtained when chromosomal DNA from mutant 7D was digested with *Hind*III, self-ligated and electroporated into MC1061. No transformants were obtained when the same amount of DNA (5 μg) was electroporated into HB101 or when the chromosomal DNA was digested with *Sma*I. Likewise, for mutant 9E, only the chromosomal DNA digested with *Hind*III, self-ligated and electroporated into MC1061 resulted in transformants. Two transformants were obtained. No transformants were obtained when HB101 was used or when the DNA was digested with *Sma*I or *Sph*I. These data indicate the importance of the genotype of the *E.coli* strain used, on the ability to rescue *erm*-containing plasmids. The reasons why no transformants were obtained when HB101 was used as the host cell or why no transformants were recovered when the DNA was initially digested with enzymes other than *Hind*III are not known.

Plasmid DNA was isolated from the transformants obtained with mutant 7D (pJM1) and mutant 9E (pJM2) DNA. Examination of these plasmids by restriction digestion and gel electrophoresis indicated that an identical plasmid was present in 7 of the 8 transformants which contained DNA rescued from mutant 7D and an identical plasmid was present in the 2 transformants which contained DNA rescued from mutant 9E. Figures 3.20A and 3.20B show the plasmids after *Bam*HI-*Hind*III digestion, respectively. As expected, from analysis of the linear maps shown in Figure 3.18A and B, *Bam*HI-*Hind*III digestion of the rescued plasmids resulted in 3 bands. These represented the 2.4 kb *Hind*III fragment, (Figures 3.20A, lane 2 and 3.20B, lane 3 lower arrows), and the 6.8 kb *Bam*HI fragment (Figures 3.20A, lane 2, middle arrow and 3.20B, lane 3, upper arrow) which were common to both plasmids because they were present within the transposon. The *Bam*HI-*Hind*III fragments which included the remaining part of the transposon (approximately 3.5 kb) and the flanking chromosomal


A
1 2 3 4 5 6 7 8 9 10 11
kb
10
7
5
3
1.6
0.6

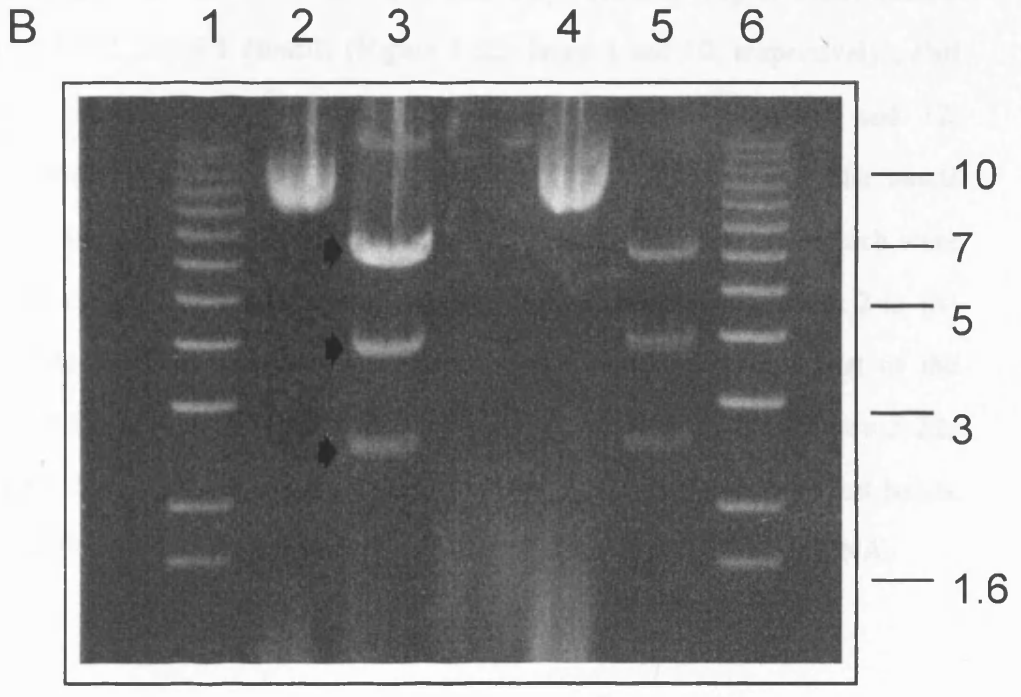

B
1 2 3 4 5 6
10
7
5
3
1.6

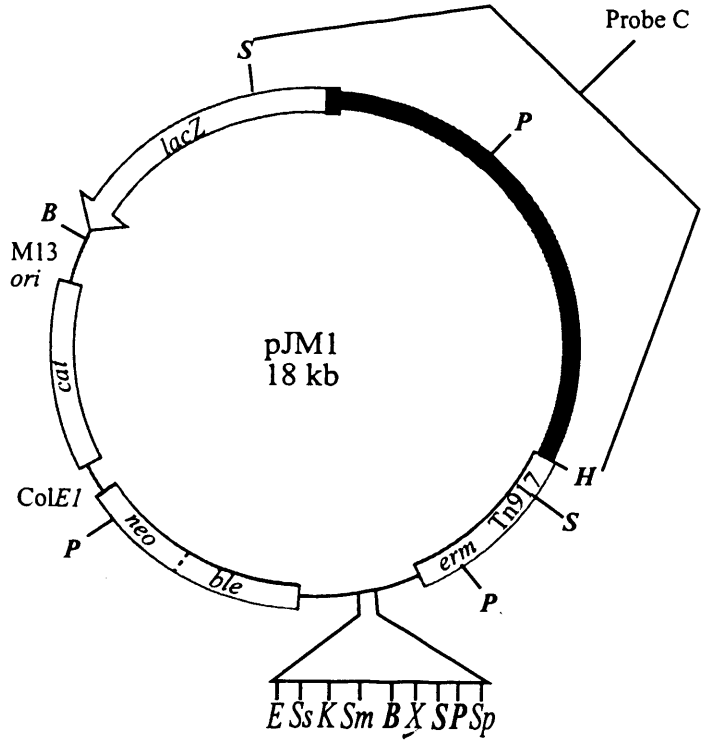
Figure 3.20A. Plasmids isolated from the 8 *E. coli* MC1061 transformants obtained with mutant 7D DNA. Electrophoretic patterns of *Bam*HI-*Hind*III-digested plasmids isolated from 8 *E. coli* MC1061 transformants. These transformants were obtained when *E. coli* MC1061 was electroporated with *Hind*III-digested, self-ligated chromosomal DNA from mutant 7D, (lanes 2 - 9). Lanes 1 and 11 represent 1 kb ladder while lane 10 represents *Bam*HI-*Hind*III digested pLTV3.

Figure 3.20B. Plasmids isolated from the 2 *E. coli* MC1061 transformants obtained with mutant 9E DNA. Electrophoretic patterns of *Bam*HI-*Hind*III-digested plasmids isolated from 2 *E. coli* MC1061 transformants (lanes 3 and 5). These transformants were obtained when *E. coli* MC1061 was electroporated with *Hind*III-digested, self-ligated chromosomal DNA from mutant 7D. Lanes 1 and 6 represent 1 kb ladder while lanes 2 and 4 represent undigested plasmids.

DNA were different sizes in each plasmid: approximately 9 kb in the plasmid containing DNA from mutant 7D (Figure 3.20A, lane 2, upper arrow) and approximately 4 kb in mutant 9E (Figure 3.20B, lane 3, middle arrow). This suggested that 5.5 kb of listerial DNA had been rescued in pJM1 while 0.5 kb of listerial DNA had been rescued in pJM2.

The recovery of plasmids, from mutant 7D and 9E, which contained transposon-flanking chromosomal DNA had been performed successfully. These plasmids were designated pJM1 (from mutant 7D) and pJM2 (from mutant 9E) and the maps are shown in Figure 3.21. Probe C and D indicated in these diagrams were used in the dot blots described below.

After large scale preparation of pJM1 and pJM2, restriction digests with other enzymes were performed to confirm the position of restriction sites. The results are shown in Figure 3.22. The positions of the *Bam*HI, *Hind*III, *Pst*I and *Sal*I sites were confirmed. Using the maps of pJM1 and pJM2 shown in Figure 3.22 where the approximate locations of the *Bam*HI, *Hind*III, *Pst*I and *Sal*I sites are indicated in bold, it was possible to determine the number of bands expected after digesting pJM1 and pJM2 with *Bam*HI (Figure 3.22, lanes 2 and 8, respectively), *Hind*III (Figure 3.23, lanes 3 and 9, respectively), *Bam*HI-*Hind*III (Figure 3.22, lanes 4 and 10, respectively), *Pst*I (Figure 3.22, lanes 5 and 11, respectively), *Sal*I (Figure 3.22, lanes 6 and 12, respectively) and *Pst*I-*Sal*I(Figure 3.22, lanes 7 and 13, respectively). The bands which represented the fragments of DNA from within the transposon, and which were common to both pJM1 and pJM2 are indicated by arrows (Figure 3.22, lanes 2 to 13) while the bands which represented the fragment of DNA containing a part of the transposon and the flanking chromosomal DNA are represented by (*) (Figure 3.22, lanes 2 to 13). In certain digests (Figure 3.22, lanes 3, 4 and 10) unexpected bands were observed. These bands were most likely due to partial digestion of the DNA.


Probe C
S
P
lacZ
B
M13
ori
cat
pJM1
18 kb
ColE1
P
neo
ble
erm
Tn917
H
S
P
E Ss K Sm B X S P Sp

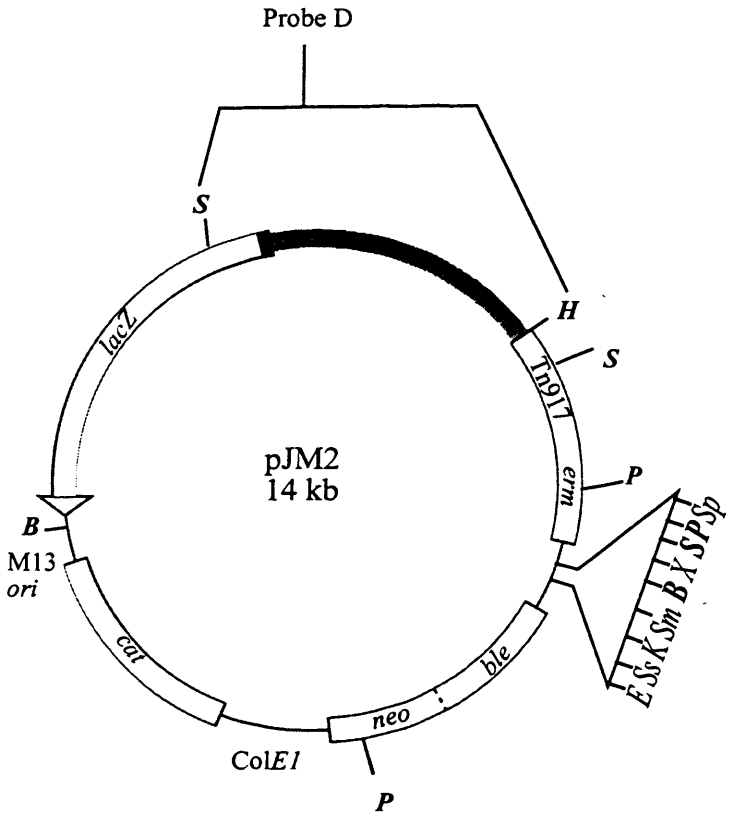

Probe D
S
lacZ
H
S
Tn917
erm
P
pJM2
14 kb
B
M13
ori
cat
neo
ble
ColE1
P
E Ss K Sm B X S P Sp

Figure 3.21. Map of plasmid pJM1 rescued from mutant 7D and pJM2 rescued from mutant 9E. Structures of rescued plasmids pJM1 and pJM2. Listerial chromosomal DNA is indicated by the thick solid line in each plamsid. Restriction sites are as follows: *B, BamHI; E, EcoRI; H, HindIII; K, KpnI; P, PstI; S, SalI; Sm, SmaI; Sp, SphI; X, XbaI.* *Bam*HI, *Hind*III, *Pst*I and *Sal*I sites are indicated in bold. Probes C and D which are used in the dot blot shown in Figure 3.23 are indicated.

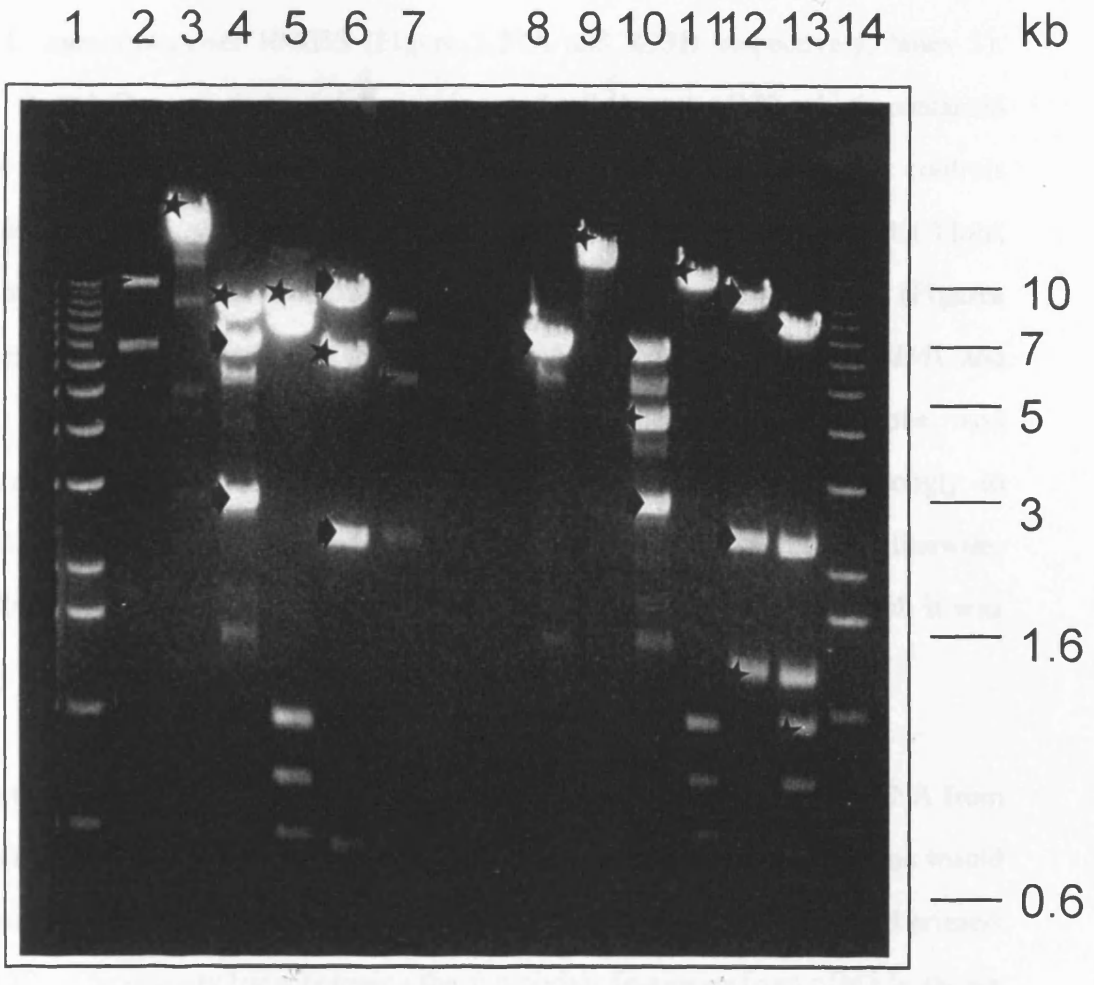

1 2 3 4 5 6 7 8 9 10 11 12 13 14
kb
10
7
5
3
1.6
0.6

Figure 3.22. Restriction digests of plasmids pJM1 and pJM2. Plasmids pJM1 and pJM2 were digested with *Bam*HI (lane 2 and 8, respectively), *Hind*III (lane 3 and 9), *Bam*HI-*Hind*III (lane 4 and 10), *Pst*I (lane 5 and 11), *Sal*I (lane 6 and 12), and *Pst*I-*Sal*I (lane 7 and 13) and analysed on a 0.7 % (w/v) agarose gel. Arrows indicate the bands which are common to both plasmids as they occur internally within the *Tn*917-pLTV3 region of the plasmids while (*) indicates the band which are different sizes in both plasmids as they contain the chromosomal DNA immediately flanking the transposon in addition to a fragment of the transposon (see restriction maps in Figure 3.21). Lanes 1 and 14 represent the 1 kb ladder and approximate sizes are indicated on the right.

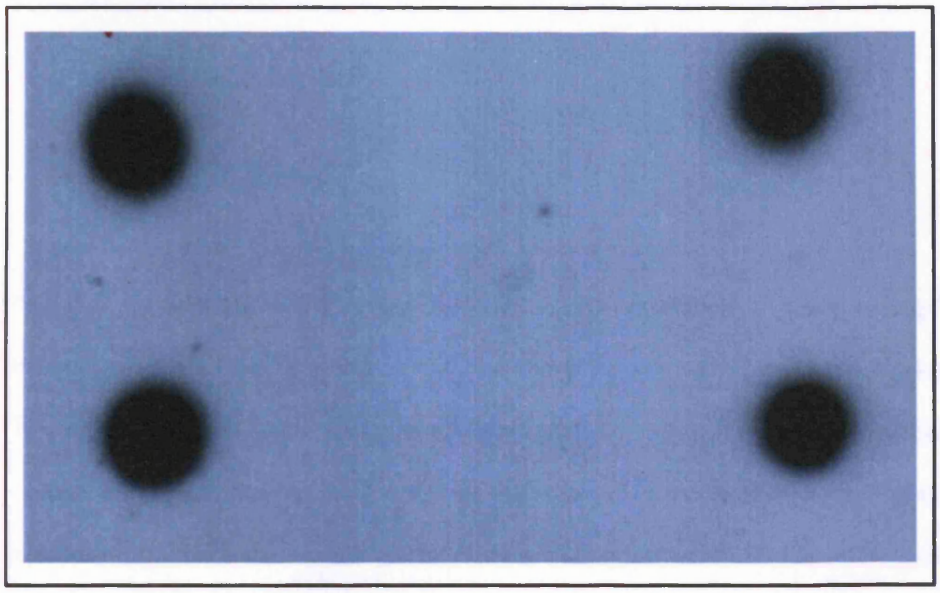
However, from the digests performed and shown in Figure 3.22 confirmation of the maps given for the rescued plasmids, pJM1 and pJM2 was made.

To further confirm that both these plasmids, pJM1 and pJM2, contained listerial chromosomal DNA, dot blots were performed, at high stringency, using Probe C from pJM1 (a 5 kb *Hind*III-*Sal*I fragment; Figure 3.21) or Probe D from pJM2 (a 1 kb *Sal*I-*Hind*III fragment; Figure 3.21) to hybridise chromosomal DNA from the wild type strain, *L. monocytogenes* 10403S (Figure 3.23A and 3.23B respectively, lanes 3). Probes C and D were derived from regions of pJM1 and pJM2 which contained putative listerial DNA. The plasmids pJM1 and pJM2 were used as positive controls while pBluescript^{SK} was used as a negative control. As evident from the dot blots, strong hybridisation occurred between the wild type DNA and both probes (Figures 3.23A and B, lane 3) confirming that listerial DNA is present in plasmids pJM1 and pJM2. As expected, no hybridisation occurred between either probe and pBluescript^{SK} (Figures 3.23A and B, lane 2). Probe C hybridised strongly to pJM1(Figure 3.23A, lane 1), the plasmid from which it was derived and likewise, Probe D hybridised to pJM2 (Figure 3.23B, lane 1) the plasmid from which it was derived.

The next stage of the investigation, involved the subcloning of a fragment of DNA from pJM1 which contained flanking chromosomal DNA, into pBluescript^{SK}. This would enable sequencing of the chromosomal DNA using the pBluescript^{SK} universal primers T3 and T7. The strategy for subcloning the appropriate fragments from pJM1 is shown in Figure 3.24. Due to the relatively small size of the chromosomal DNA in plasmid pJM2, sequencing was performed directly, using plasmid pJM2 as a template and primers based on *Tn*917. This is discussed in more detail in section 3.8 below.

A 5 kb *Hind*III-*Sal*I fragment from pJM1 was cloned into *Hind*III-*Sal*I digested pBluescript^{SK} to produce a plasmid designated pJM6. Plasmid pJM6 was then

A 1 2 3



B 1 2 3

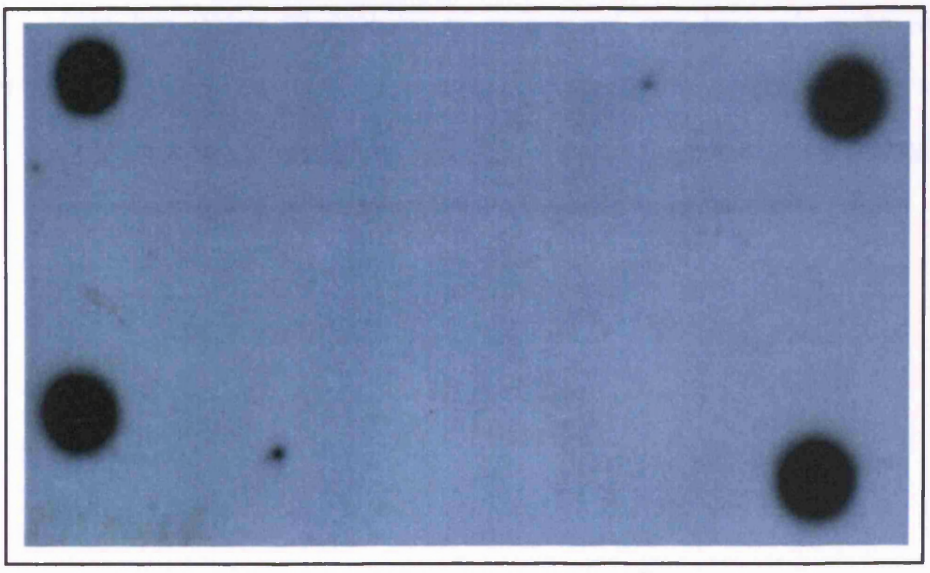


Figure 3.23. Dot blot analysis of *L. monocytogenes* 10403S (wild type) listerial DNA using a probe derived from pJM1 (Figure 3.23A) and a probe derived from pJM2 (Figure 3.23B). 1-2 μg of chromosomal DNA from *L. monocytogenes*, 10403S (wild type) was spotted onto nylon membrane (Figures 3.23A and b, lane 3) in addition to 1-2 μg of pJM1 (Figure 3.23A, lane 1), 1-2 μg of pJM2 (Figure 3.23B, lane 1) and 1-2 μg of pBluescript^{SK} (Figures 3.23A and B, lane 2). Probe C, derived from pJM1 and Probe D, derived from pJM2 were labelled with [α-^{32}P] and used for hybridising (Figures 3.23A and B, respectively) under conditions of high stringency. The membranes were washed 3 times in 0.1 X SSC, 0.1 % (w/v) SDS at 68°C for 20 min. Each sample was tested in duplicate, indicated by the two rows under each lane number.

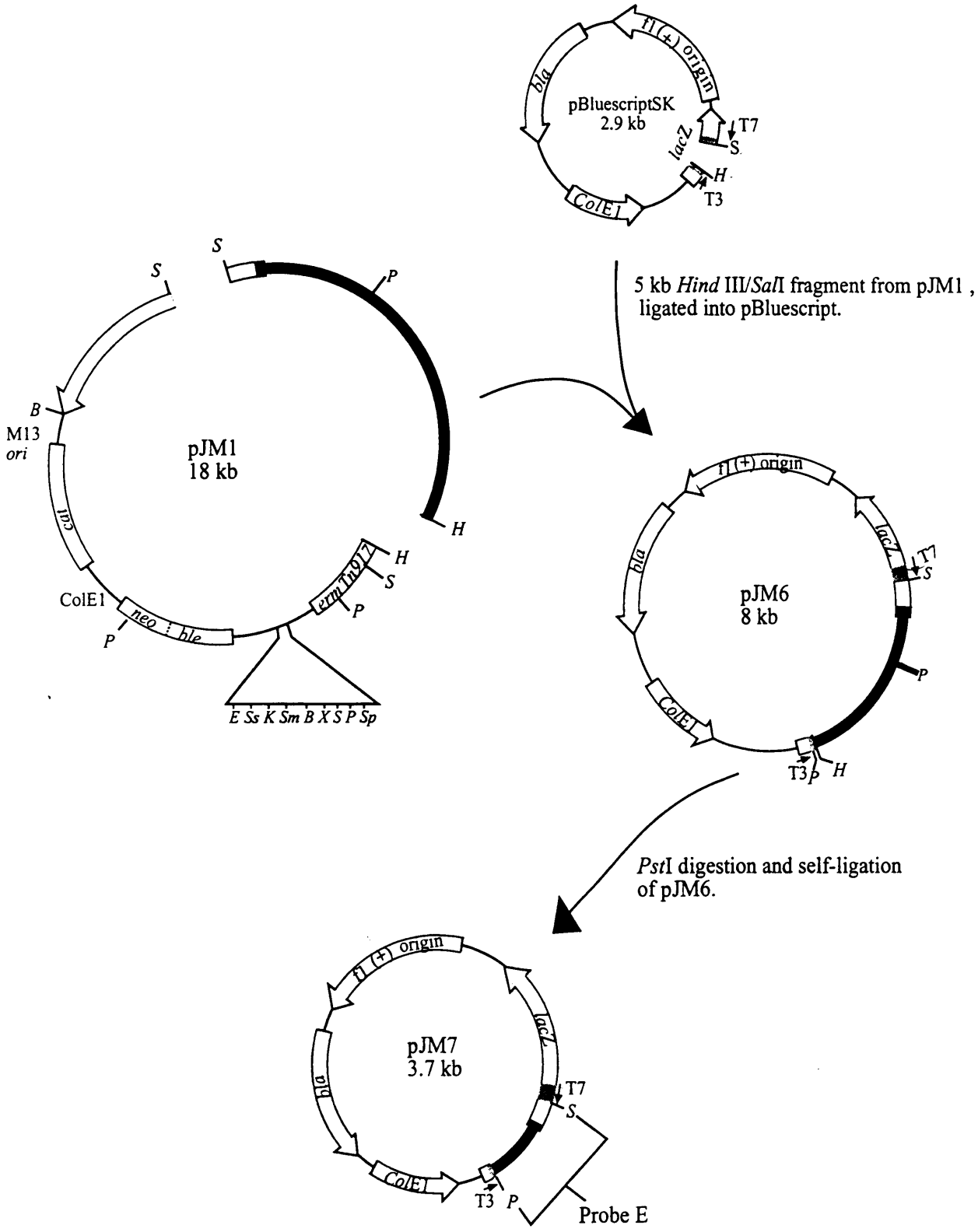

pBluescriptSK
2.9 kb
f1 (+) origin
bla
lacZ
T7
S
H
T3
ColE1
5 kb Hind III/SalI fragment from pJM1 , ligated into pBluescript.
pJM1
18 kb
M13
ori
B
S
P
H
cat
ermBpZ
ColE1
neo : ble
E Ss K Sm B X S P Sp
pJM6
8 kb
PstI digestion and self-ligation of pJM6.
pJM7
3.7 kb
Probe E

Figure 3.24 Strategy for subcloning a chromosomal DNA-containing fragment of pJM1 into pBluescript^{SK}. Diagram showing the approach taken to subclone a 5 kb *Hind*III-*Sal*I fragment from pJM1 into pBluescript^{SK}. The plasmid made up of pBluescript^{SK} and the 5 kb *Hind*III-*Sal*I fragment from pJM1 was designated pJM6. Plasmid pJM6 was then digested with *Pst*I and incubated overnight at 14°C with T4 DNA ligase. Digestion of the ligation mixture, prior to electroporation, with *Hind*III prevented recovery of *Hind*III-containing pJM6 and lead to recovery of another plasmid, designated pJM7, which did not contain a *Hind*III site. pJM7 consisted of pBluescript^{SK} and an 800 bp fragment containing a part of the transposon and the listerial DNA immediately flanking the site of transposon insertion. Both these plasmids were used for sequencing (see section 3.6 below). The positions of the T3 and T7 primer sites are indicated. The listerial DNA is represented by the solid black line while the solid grey boxes seen in pBluescript, pJM6 and pJM7 represent the multiple cloning site within the *lacZ* gene of pBluescript. Probe E which was used in Figure 3.26 is shown.

digested with *Pst*I and incubated overnight at 14°C with T4 DNA ligase. The ligation mixture was digested with *Hind*III prior to electroporation into *E. coli* DH5α as this would prevent recovery of any pJM6 molecules which were present. This procedure allowed the recovery of plasmid pJM7; pBluescript^{SK} containing an 800 bp *Pst*I-*Sal*I fragment of DNA from pJM1. This *Pst*I-*Sal*I fragment contained the listerial DNA immediately flanking the transposon insertion. The maps of pJM6 and pJM7 are shown in Figure 3.24 where the 800 bp *Pst*I-*Sal*I fragment is indicated as Probe E. Probe E was used in the dot blot described below.

Confirmation of the successful recovery of pJM6 and pJM7 was achieved by performing restriction digests on isolated plasmids. *Pst*I-digested pJM6 resulted in 2 bands, as expected (Figure 3.25, lane 4). These bands represented the 5 kb band which contained the chromosomal DNA and part of pBluescript^{SK} and the smaller band (approximately 2.8 kb) which consisted of the remainder of pBluescript^{SK}. Lane 5 shows the results obtained after *Pst*I-*Sal*I digestion of pJM7. The smaller band (approximately 800 bp) represent the fragment of DNA which contained the chromosomal DNA immediately flanking the transposon and a part of the transposon while the larger band (approximately 3 kb) represented the whole of pBluescript^{SK}. The unexpected high molecular weight bands in lanes 4 and 5 are most likely due to partial digestion of the plasmids. *Hind*III-*Sal*I-digested pJM1 and *Hind*III-*Sal*I-digested pBluescript^{SK} are shown in lanes 2 and 3, respectively.

Dot blots were performed, under highly stringent conditions, to confirm that the fragments subcloned into pBluescript^{SK} from pJM1 contained listerial DNA. Figure 3.26 shows the results obtained when the 800 bp *Pst*I-*Sal*I fragment from pJM7 (Probe E) was used to hybridise pJM1 (lane 1), pJM7 (lane 2), pBluescript^{SK} (lane 3) and wild type DNA (lane 4). The results showed hybridisation of Probe E to pJM1, pJM7 and to wild type DNA but not to pBluescript^{SK}, thus indicating that the listerial DNA

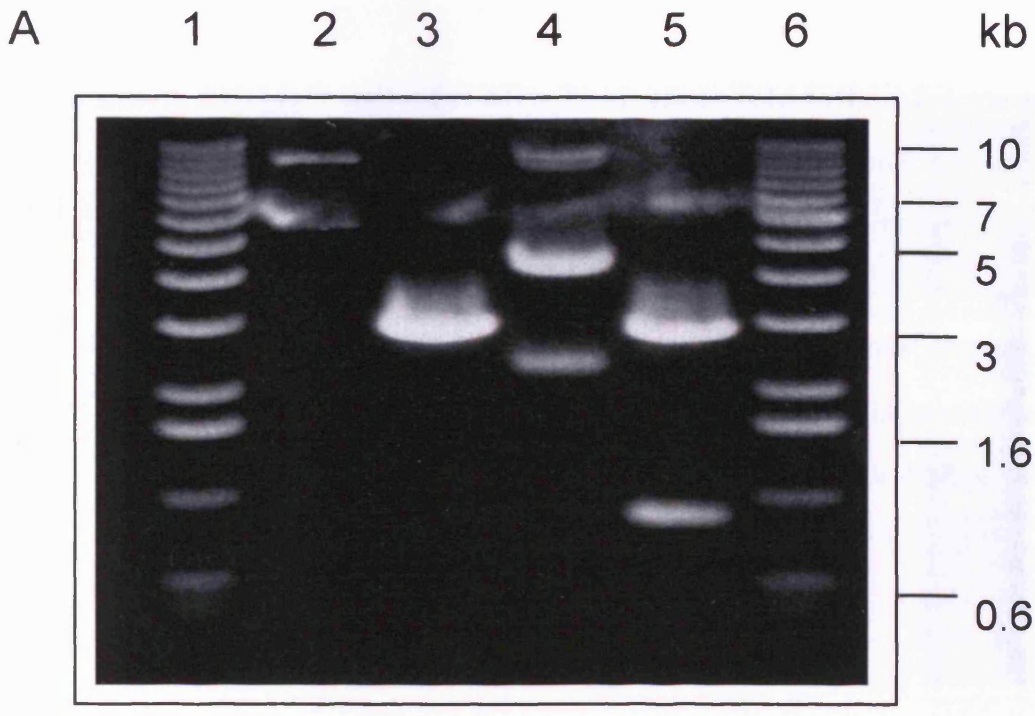

A
1
2
3
4
5
6
kb
10
7
5
3
1.6
0.6

Figure 3.25. Restriction digests of pJM6 and pJM7. Plasmids pJM6 digested with *Pst*I (lane 4) and pJM7 digested with *Pst*I-*Sal*I (lane 5) were analysed on a 0.7% (w/v) agarose gel. *Hind*III-*Sal*I-digested pJM1 and *Hind*III-*Sal*I-digested pBluescript^{SK} are shown in lanes 2 and 3, respectively. Lanes 1 and 6 represent the 1 kb ladder and approximate sizes are indicated on the right.

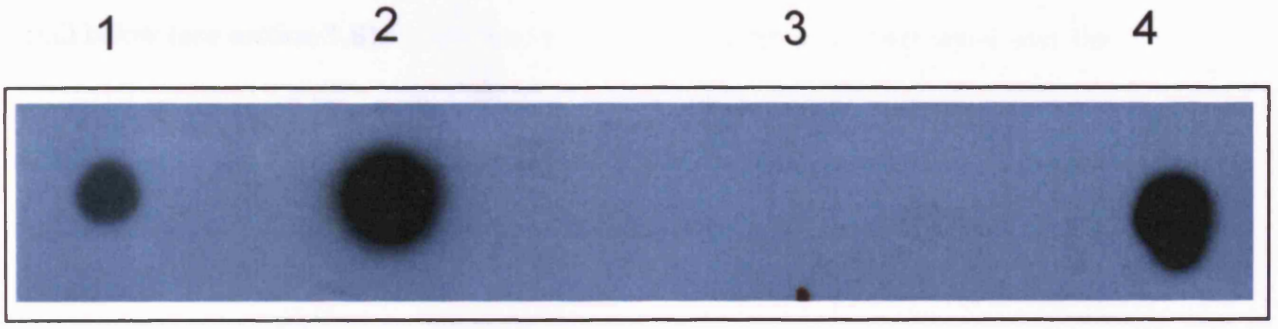

1
2
3
4

Figure 3.26. Dot blot analysis of wild type listerial DNA using a probe derived from pJM7. 1-2 μg of chromosomal DNA from *L. monocytogenes*, 10403S (wild type) was spotted onto nylon membrane (lane 4) in addition to 1-2 μg of pJM1 (lane 1), 1-2 μg of pJM7 (lane 2) and 1-2 μg of pBluescript^{SK} (lane 3). Probe E, derived from pJM7 was labelled with [α-^{32}P] and used for hybridising under conditions of high stringency. The membrane was washed 3 times in 0.1 X SSC, 0.1 % (w/v) SDS at 68°C for 20 min.

immediately flanking the transposon had been successfully subcloned into pBluescript^{SK} for sequencing.

Thus, plasmids containing chromosomal DNA flanking the *erm*-proximal end of the transposon in mutant 7D and mutant 9E were successfully rescued into *E.coli* MC1061 and were designated pJM1 and pJM2, respectively. Fragments of pJM1 were subcloned into pBluescript^{SK} to produce plasmids pJM6 and pJM7 which would be used in the future for sequencing (see section 3.6 below). Sequencing of the flanking chromosomal DNA in pJM2 involved a different approach which is discussed in more detail below (see section 3.8).

3.6 Sequence analysis of chromosomal DNA flanking the *erm*-proximal end of the transposon in mutant 7D

Approximately 800 bp of DNA containing part of the transposon and the fragment of listerial DNA immediately flanking the transposon was successfully subcloned into pBluescript^{SK} to produce plasmid pJM7 (Figure 3.27).

DNA sequencing of both strands of the entire insert in pJM7 was performed three times using the Automated Dye Deoxy method. Using the universals primers T3 and T7 (Short *et al.*, 1988), the complete sequence of the insert was determined and the nucleotide sequence of the region from the inverse repeat within the tranpsoson to the end of the listerial DNA is shown in Figure 3.28A. This sequence was searched against nucleic acid databases and amino acid databases using BLASTN and BLASTX programs (National Centre for Biotechnology Information, Los Alamos, N.MEX) available on the internet (http//:www.ncbi.nlm.nih.gov/cgi-bin/BLAST/). The results indicated that the listerial DNA fragment (approximately 500 bp) could be divided into two main areas each having significant nucleotide sequence homology to different genes or parts of genes. The region from nucleotides 38 to 335 showed 80% homology at the nucleotide level to a region designated the promoter of the listerial *flaA* gene (Dons *et al.*, 1992). The region from nucleotide 341 to 540, designated ORFA, showed 55% identity at the amino acid level to a gene named *cheV* of *B. subtilis* (Fredrick & Helmann, 1994) which is involved in bacterial chemotaxis. This is shown diagrammatically in Figure 3.28B. Sequence alignments and discussion of these homologies are given in the Figures which follow .

The sequence of region 341 to 550 provided evidence for the occurrence of a new listerial gene which had homology to *cheV* of *B. subtilus*. Further DNA sequencing and comparisons were made and are discussed in more detail in section 3.6.2 below.

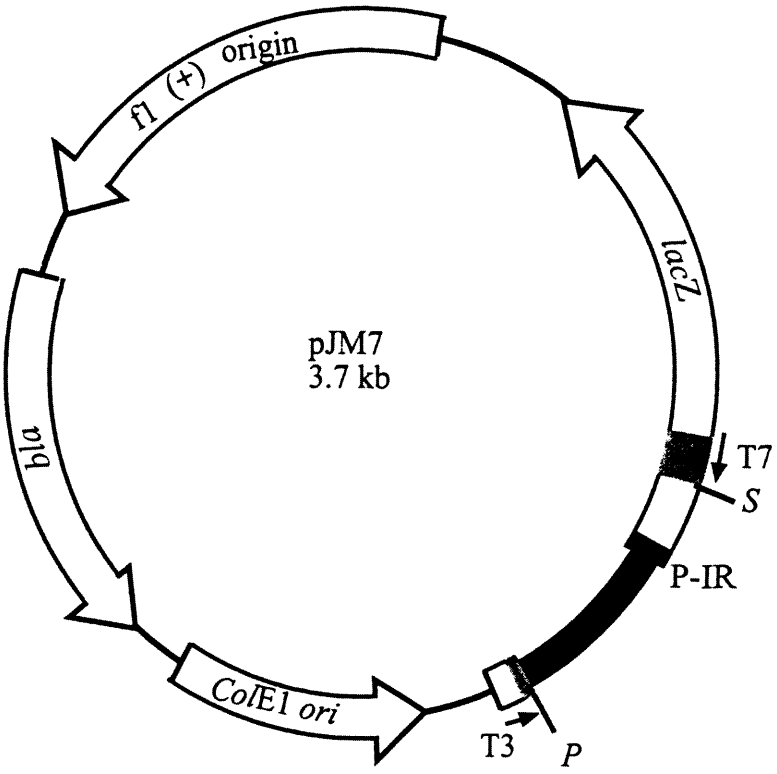
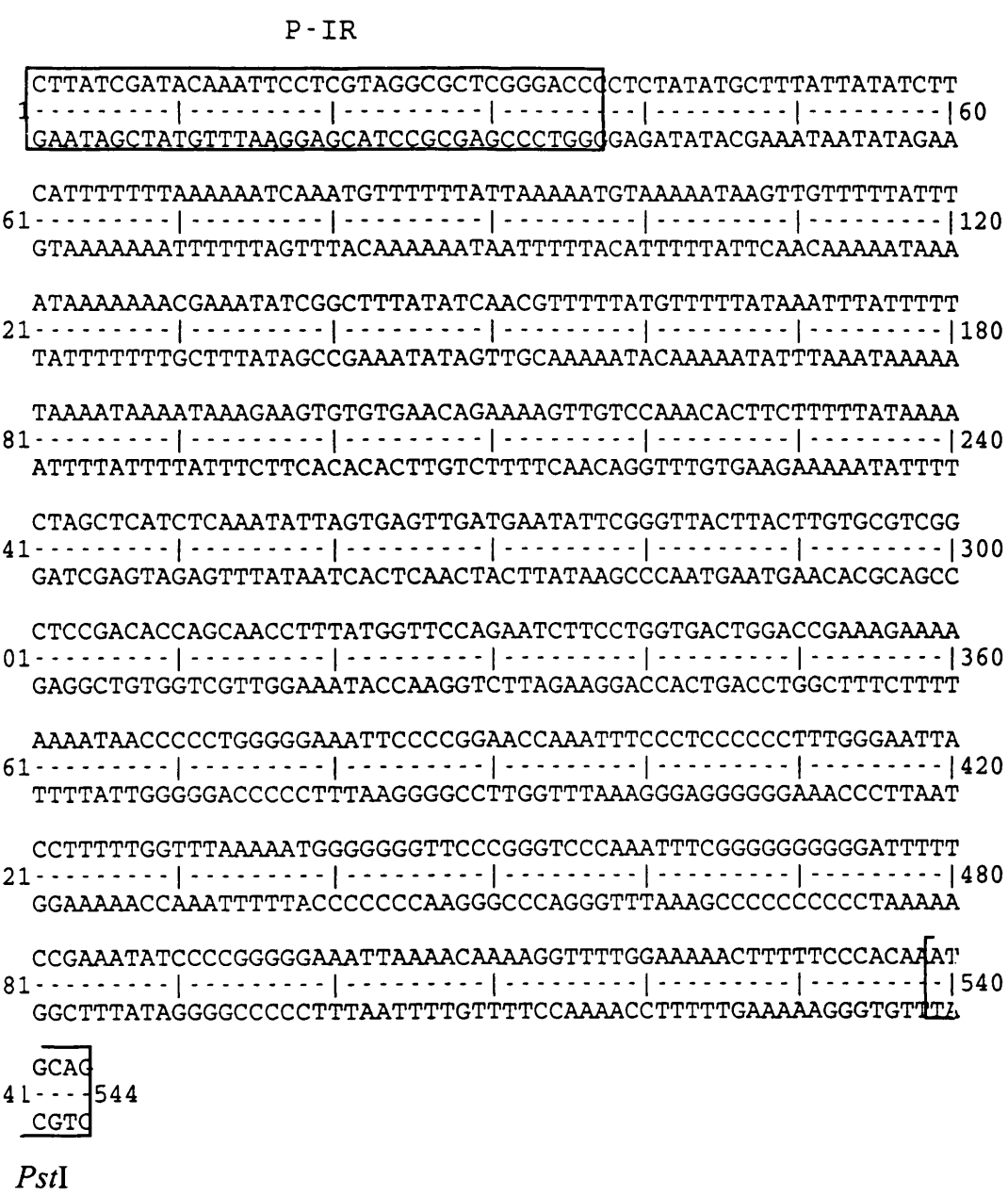

f1 (+) origin
lacZ
T7
S
P-IR
T3
P
ColE1 ori
bla
pJM7
3.7 kb

Figure 3.27. Map of pJM7. Detailed map of pJM7 constructed as described diagrammatically in Figure 3.24. Essentially pJM7 is pBluescript^{SK} containing an 800 bp *Hind*III-*Sal*I fragment of listerial DNA and part of *Tn*917 from mutant 7D. This fragment immediately flanks the *erm*-proximal end of the transposon insertion. The positions of the T3 and T7 primer sites are indicated.

A


P-IR
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GAATAGCTATGTTTAAGGAGCATCCGCGAGCCCTGGGGAGATATACGAAATAATATAGAA
CATTTTTTTAAAAAATCAAATGTTTTTTATTAAAAATGTAAAAATAAGTTGTTTTTATTT
GTAAAAAAATTTTTTAGTTTACAAAAAATAATTTTTACATTTTTATTCAACAAAAATAAA
ATAAAAAAACGAAATATCGGCTTTATATCAACGTTTTTATGTTTTTATAAATTTATTTTT
TATTTTTTTGCTTTATAGCCGAAATATAGTTGCAAAAATACAAAAATATTTAAATAAAAA
TAAAATAAAATAAAGAAGTGTGTGAACAGAAAAGTTGTCCAAACACTTCTTTTTATAAAA
ATTTTATTTTATTTCTTCACACACTTGTCTTTTCAACAGGTTTGTGAAGAAAAATATTTT
CTAGCTCATCTCAAATATTAGTGAGTTGATGAATATTCGGGTTACTTACTTGTGCGTCGG
GATCGAGTAGAGTTTATAATCACTCAACTACTTATAAGCCCAATGAATGAACACGCAGCC
CTCCGACACCAGCAACCTTTATGGTTCCAGAATCTTCCTGGTGACTGGACCGAAAGAAAA
GAGGCTGTGGTCGTTGGAAATACCAAGGTCTTAGAAGGACCACTGACCTGGCTTTCTTTT
AAAATAACCCCCTGGGGGAAATTCCCCGGAACCAAATTTCCCTCCCCCCTTTGGGAATTA
TTTTATTGGGGGACCCCCTTTAAGGGGCCTTGGTTTAAAGGGAGGGGGGAAACCCTTAAT
CCTTTTTGGTTTAAAAATGGGGGGGTTCCCGGGTCCCAAATTTCGGGGGGGGGGATTTTT
GGAAAAACCAAATTTTTACCCCCCCAAGGGCCCAGGGTTTAAAGCCCCCCCCCCTAAAAA
CCGAAATATCCCCGGGGGAAATTAAAACAAAAGGTTTTGGAAAAACTTTTTCCCACAAAT
GGCTTTATAGGGGCCCCCTTTAATTTTGTTTTCCAAAACCTTTTTGAAAAAGGGTGTTTA
GCAC
541---544
CGTC
PstI

B

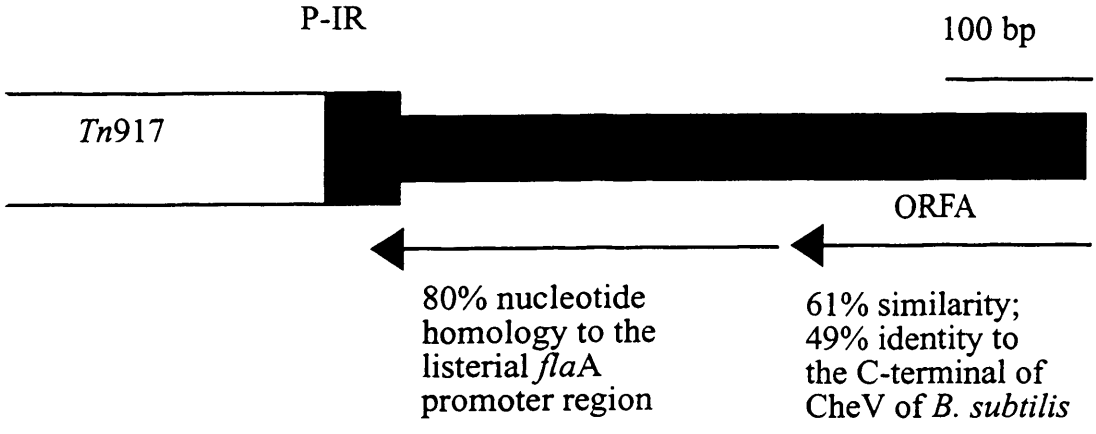

P-IR
100 bp
Tn917
ORFA
80% nucleotide homology to the listerial flaA promoter region
61% similarity; 49% identity to the C-terminal of CheV of B. subtilis

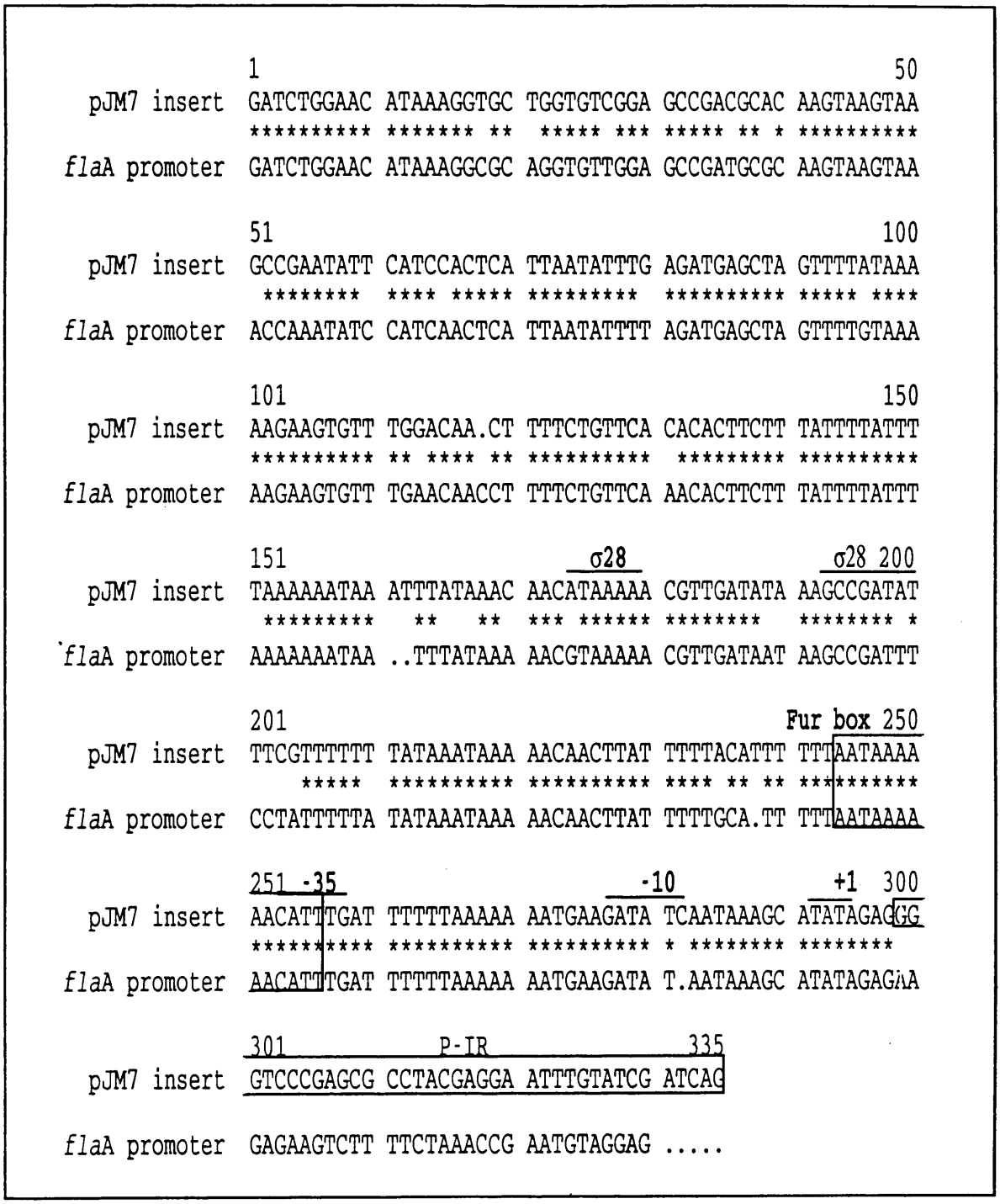
Figure 3.28A Nucleotide sequence of the insert in pJM7. The sequence of the insert in pJM7, from the *erm*-proximal inverse repeat (P-IR) to the *Pst*I site indicating the end of the insert, is shown.

Figure 3.28B. Diagrammatic representation of the listerial DNA insert in pJM7. A schematic diagram showing the chromosomal DNA flanking the erm-proximal end of transposon insertion. The 298 bp fragment immediately flanking the transposon showed significant homology to the *flaA* promoter region of *L. monocytogenes* while the 209 bp fragment further downstream (ORFA) from the transposon showed significant homology to *cheV* of B. subtilis. The arrowheads indicate the direction of transcription of these putative genes.

Figure 3.29 shows a comparison of the *flaA* promoter region (Dons *et al.*, 1992) with the nucleotide sequence of residues 1 to to 335 of the insert. The important features are highlighted and include the consensus boxes -10 and -35, the σ^{28} consensus regions and also the putative transcription initiation site of the *flaA* mRNA (+1). Noticeably, the putative ribosome binding site (rbs) is not present but presumably is present on the chromosomal DNA flanking the other side of the transposon, together with the sequence encoding the structural flagellin. This supposition was investigated and is discussed below in section 3.6.1.

Interestingly, in this study, the presence of a Fur-like box, similar to those identified upstream from iron-regulated genes of *E. coli* (de Lorenzo *et al.*, 1988) and other bacterial species (Tai & Holmes, 1988) was observed between residues 243 to 257. This 15-bp motif was identified using Genetic Computer Group programs, while looking for Fur-like box motifs (de Lorenzo *et al.*, 1988) or a Dtx-recognition sequence (Murphy *et al.*, 1978; Tai & Homes, 1988) in the listerial DNA sequence. The reason for looking for these motifs was due to the iron-related phenotype of mutant 7D. The Fur-like box is typically found upstream from iron-regulated genes of Gram-negative species, although a similar motif was found upstream from another *listerial* gene, the *kat*G gene (Haas, *et al.*, 1991). The Dtx recognition sequence was identified upstream from the *Corynebacterium diptheriae*, iron-regulated, *tox* gene (Murphy, *et al.*, 1978) and was thought to be a Gram-positive equivalent of the Fur box. This new finding of a Fur-like box upstream from *fla*A is discussed in more detail in section 4.3. No Dtx recognition sequences were identified in the *flaA* promoter region.

Figure 3.29. Comparison of region 1 to 335 of the pJM7 insert to the *flaA* promoter region from *L. monocytogenes*. Comparison of the 300 bp immediately flanking the erm-proximal end of transposition in mutant 7D with the designated promoter region of the listerial *fla*A gene (Dons *et al.*, 1992). The important features, -35, -10, σ^{28}, transcription initiation site (+1) are indicated. The transposon inverse repeat is indicated by IR. Identical nucleotides are indicated by asterisks. The Fur-like box, identified using Genetics Computer Group programs is shown.


1 50
pJM7 insert GATCTGGAAC ATAAAGGTGC TGGTGTCGGA GCCGACGCAC AAGTAAGTAA
flaA promoter GATCTGGAAC ATAAAGGCGC AGGTGTTGGA GCCGATGCGC AAGTAAGTAA
51 100
pJM7 insert GCCGAATATT CATCCACTCA TTAATATTTG AGATGAGCTA GTTTTATAAA
flaA promoter ACCAAATATC CATCAACTCA TTAATATTTT AGATGAGCTA GTTTTGTAAA
101 150
pJM7 insert AAGAAGTGTT TGGACAA.CT TTTCTGTTCA CACACTTCTT TATTTTATTT
flaA promoter AAGAAGTGTT TGAACAACCT TTTCTGTTCA AACACTTCTT TATTTTATTT
151 σ28 σ28 200
pJM7 insert TAAAAAATAA ATTTATAAAC AACATAAAAA CGTTGATATA AAGCCGATAT
flaA promoter AAAAAAATAA ..TTTATAAA AACGTAAAAA CGTTGATAAT AAGCCGATTT
201 Fur box 250
pJM7 insert TTCGTTTTTT TATAAATAAA AACAACTTAT TTTTACATTT TTTAATAAAA
flaA promoter CCTATTTTTA TATAAATAAA AACAACTTAT TTTTGCA.TT TTTAATAAAA
251 -35 -10 +1 300
pJM7 insert AACATTTGAT TTTTTAAAAA AATGAAGATA TCAATAAAGC ATATAGACGG
flaA promoter AACATTTGAT TTTTTAAAAA AATGAAGATA T.AATAAAGC ATATAGAGAA
301 P-IR 335
pJM7 insert GTCCCGAGCG CCTACGAGGA ATTTGTATCG ATCAG
flaA promoter GAGAAGTCTT TTCTAAACCG AATGTAGGAG .....

3.6.1 Polymerase chain reaction (PCR) amplification and sequencing of the chromosomal DNA at the *erm*-distal end of the transposon in mutant 7D

To confirm that the transposon had inserted into the flagellin encoding gene of *L. monocytogenes*, the chromosomal DNA flanking the *erm*-distal end of the transposon was sequenced. A different approach to the determination of this sequence was taken and involved PCR amplification using a primer complimentary to a fragment of DNA containing part of the *erm*-distal end of the transposon and a primer complimentary to a region of the *flaA* sequence (Dons *et al.*, 1992) which we were assuming was present at the *erm*-distal side of transposition. This approach is shown diagrammatically in Figure 3.30. The primers used were designated ERM.DIST and FLAA. Primer ERM.DIST, GGCCTTGAAACATTGGTTTAGTGGG, was based on a region of *Tn*917, 100 bp from the *erm*-distal inverse repeat (D-IR) (Shaw & Clewell, 1985) and was made in the sense direction to facilitate priming towards the flanking chromosomal DNA. Primer FLAA, GCAGCAACTGTAGAACCACTACCTA, was based on a region of the *flaA* gene *of L. monocytogenes* (Dons *et al.*, 1992), approximately 600 bp from the ribosome binding site and was made in the antisense direction to facilitate priming towards the transposon. Therefore, the predicted length of PCR product expected from ERM.DIST to FLAA was be approximately 700 bp.

The PCR reactions were performed as described previously (section 2.11) using Taq DNA polymerase and 35 cycles of: 94°C, 30 sec; 56°C, 1 min; 72°C, 2 min. The resulting PCR products were analysed on a 0.7% (w/v) agarose gel (Figure 3.31). Lane 2 shows the PCR product obtained when chromosomal DNA from *L. monocytogenes*, mutant 7D was use as the template while lane 3 shows the result obtained when *L. monocytogenes*, wild type DNA was used as a template. Not surprisingly, no PCR product was obtained when wild type DNA was used as

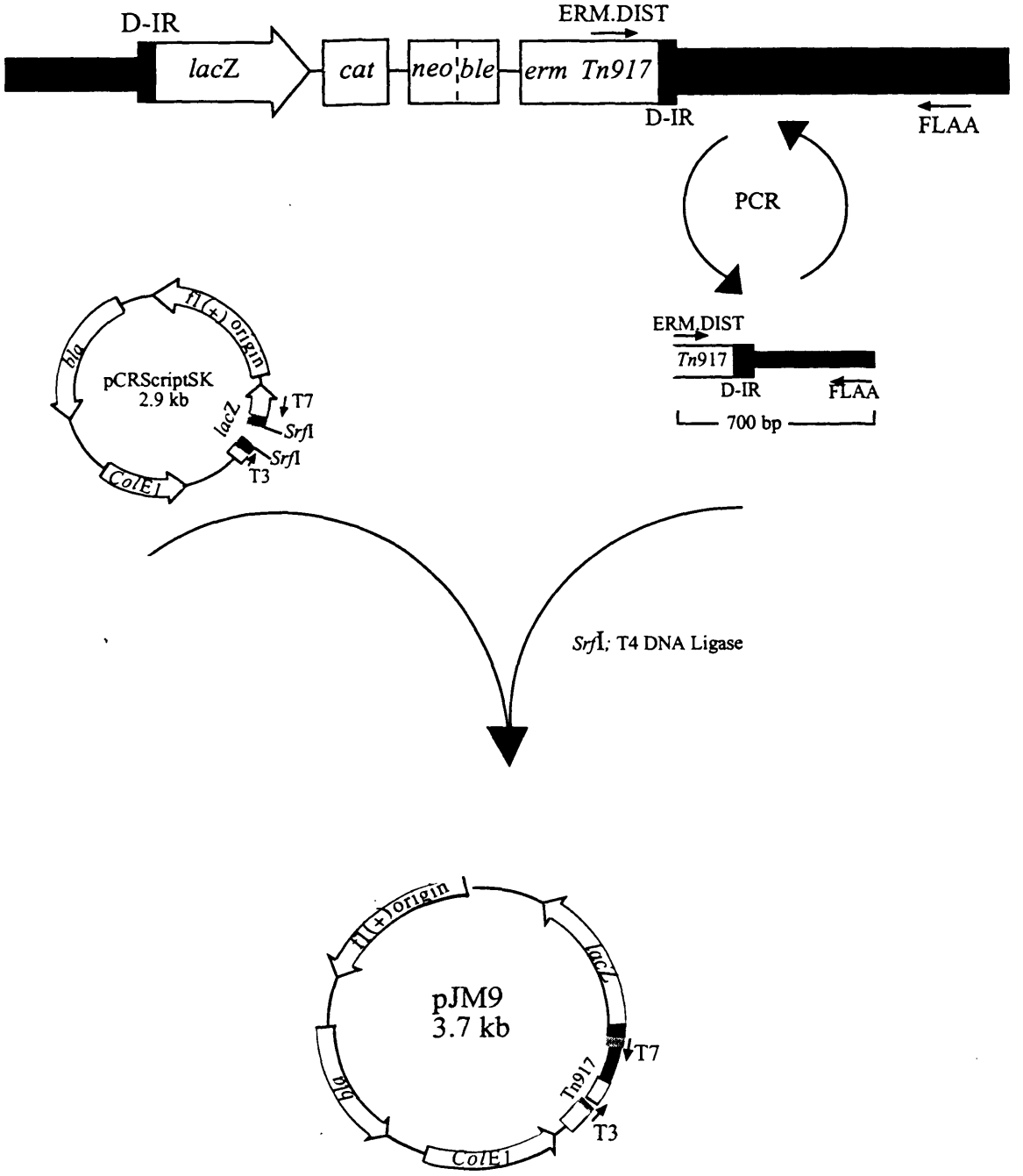

D-IR
ERM.DIST
lacZ
cat
neo
ble
erm Tn917
D-IR
FLAA
PCR
ERM.DIST
Tn917
D-IR
FLAA
700 bp
pCRScriptSK
2.9 kb
f1(+) origin
bla
lacZ
T7
SrfI
SrfI
T3
ColE1
SrfI; T4 DNA Ligase
pJM9
3.7 kb
f1(+) origin
lacZ
T7
Tn917
T3
bla
ColE1

Figure 3.30. PCR approach to sequence the *erm*-distal flanking chromosomal DNA in mutant 7D. Diagram showing the approach taken to sequence the listerial DNA flanking the *erm*-distal end of the transposon insertion. Primers ERM.DIST and FLAA were used to amplify a 700 bp fragment at the *erm*-distal end of the transposon. Using the pCRScript^{SK} Cloning Kit the PCR product produced was subcloned into the vector to produce a plasmid designated pJM9. pCRScript^{SK} is essentially pBluescript^{SK} with an SrfI site in the multiple cloning site (indicated by the grey box). Two alternate forms of pJM9 could have been produced, depending on the orientation of insertion of the PCR product into pCRScript^{SK}. However only form form is shown in the diagram opposite. pJM9 was used for sequencing the *erm*-distal flanking DNA with T3 and T7 primers.

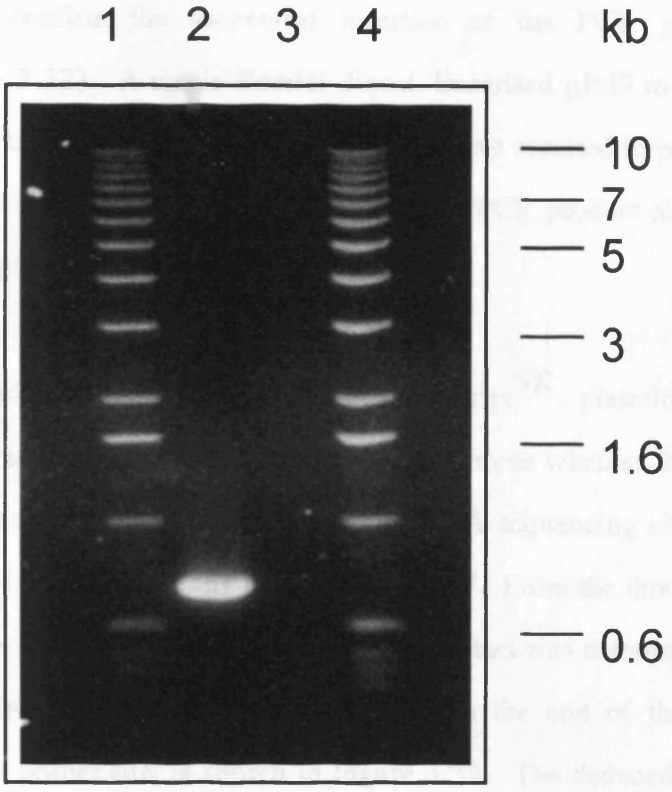

1
2
3
4
kb
10
7
5
3
1.6
0.6

Figure 3.31. Gel electrophoresis of PCR products representing the chromosomal DNA at the *erm*-distal side of transposition. *L. monocytogenes* mutant 7D (lane 2) and 10403S (wild type; lane 3) genomic DNA was used as templates for amplification with ERM.DIST and FLAA primers. *Taq* DNA polymerase was used according to manufacturer's instructions (GIBCO-BRL). Lanes 1 and 4 represent 1 kb ladder. The samples were analysed on a 0.7% (w/v) agarose gel.

a template because the wild type DNA lacks the ERM.DIST complimentary sequence within the transposon.

Using the method provided with the pCRScript^{SK} Cloning Kit (Stratagene), the fragment was firstly polished using *pfu* DNA polymerase at 72^{o}C for 30 min to ensure the PCR product was blunt-ended. The PCR product was cloned into the *Srf*I site of the vector to produce a plasmid designated pJM9. Restriction digests of this plasmid were performed to confirm the successful insertion of the PCR product into pCRScript^{SK} (Figure 3.32). A single *Bam*HI digest, linearised pJM9 to give a band of approximately 3.7 kb (lane 3) while a *Bam*HI-*Not*I digest resulted in release of the 700 bp insert from the vector (lane 4). For comparison the PCR product alone (lane 2) and *Bam*HI-digested pCRScript^{SK} (lane 5) are shown.

Having successfully cloned the PCR product into pCRScript^{SK}, plasmid pJM9 was used as a template for sequencing the 700 bp insert to determine whether the *flaA* gene was flanking the *erm*-distal end of the transposon. DNA sequencing of both DNA strands was performed three times using primers T3 and T7. From the three sequences obtained, the complete nucleotide sequence of the PCR product was determined and the nucleotide sequence from the *erm*-distal inverse repeat to the end of the insert, as indicated by the FLAA primer site, is shown in Figure 3.33. The deduced amino acid sequence, with homology to the FlaA sequence is shown below the nucleotide sequence and an alignment of this amino acid sequence with the previously published flagellin amino acid sequence (Dons *et al.*, 1992) is shown in Figure 3.34.

It was concluded that the transposon had inserted into the *flaA* gene *of L. monocytogenes* at a site between the transcription initiation site (TATA; +1) and the ribosome binding site (rbs). This is shown diagrammatically in Figure 3.35.

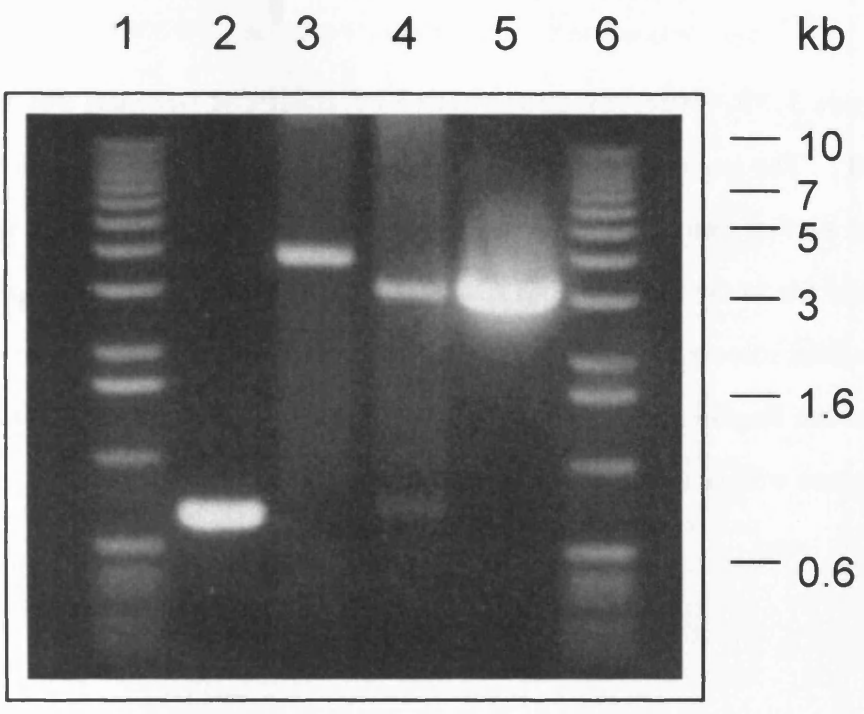

1
2
3
4
5
6
kb
10
7
5
3
1.6
0.6

Figure 3.32. Restriction digests of plasmid pJM9; essentially pCRScript^{SK} containing the PCR product shown in Figure 3.31. Plasmid pJM9 was digested with *Bam*HI (lane 3) or *Bam*HI-*Not*I (lane 4) and analysed on a 0.7% (w/v) agarose gel. The PCR product alone (lane 2) and *Bam*HI-digested pCRScript^{SK} (lane 5) were included to enable size comparisons. Lanes 1 and 6 represent 1 kb ladder.

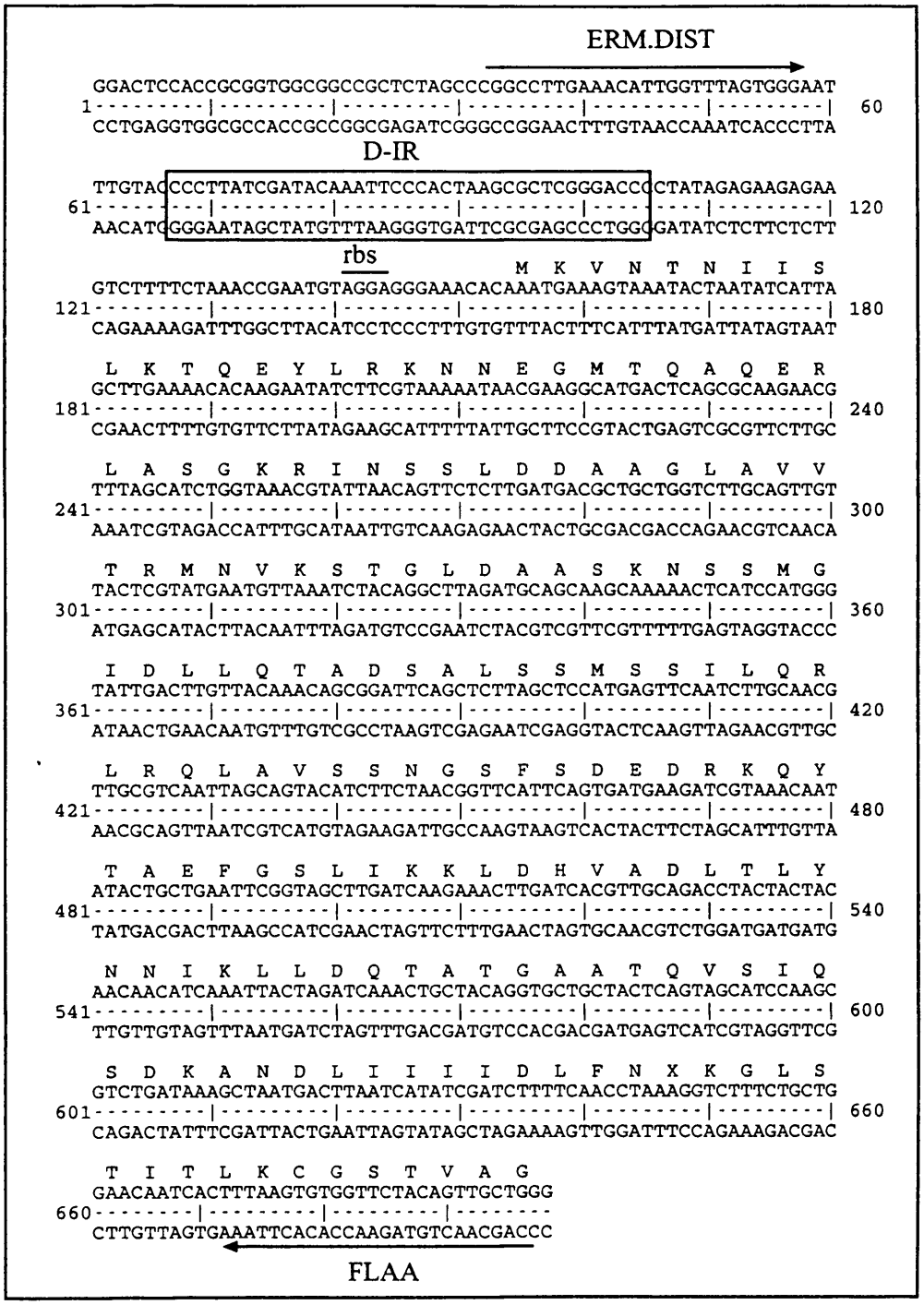

ERM.DIST
D-IR
rbs
M K V N T N I I S
L K T Q E Y L R K N N E G M T Q A Q E R
L A S G K R I N S S L D D A A G L A V V
T R M N V K S T G L D A A S K N S S M G
I D L L Q T A D S A L S S M S S I L Q R
L R Q L A V S S N G S F S D E D R K Q Y
T A E F G S L I K K L D H V A D L T L Y
N N I K L L D Q T A T G A A T Q V S I Q
S D K A N D L I I I I D L F N X K G L S
T I T L K C G S T V A G
FLAA

Figure 3.33. Nucleotide sequence of the PCR product shown in Figure 3.31. The sequence of the PCR product representing listerial DNA from the *erm*-distal end of transposon insertion in mutant 7D. The region from the ERM.DIST primer site to the FLAA primer site is shown. Also indicated are the positions of the *erm*-distal inverse repeat (D-IR) and the putative ribosome binding site (rbs) upstream from the flagllin encoding sequence (nucleotide 156). The deduced amino acid sequence with is shown above the nucleotide sequence.

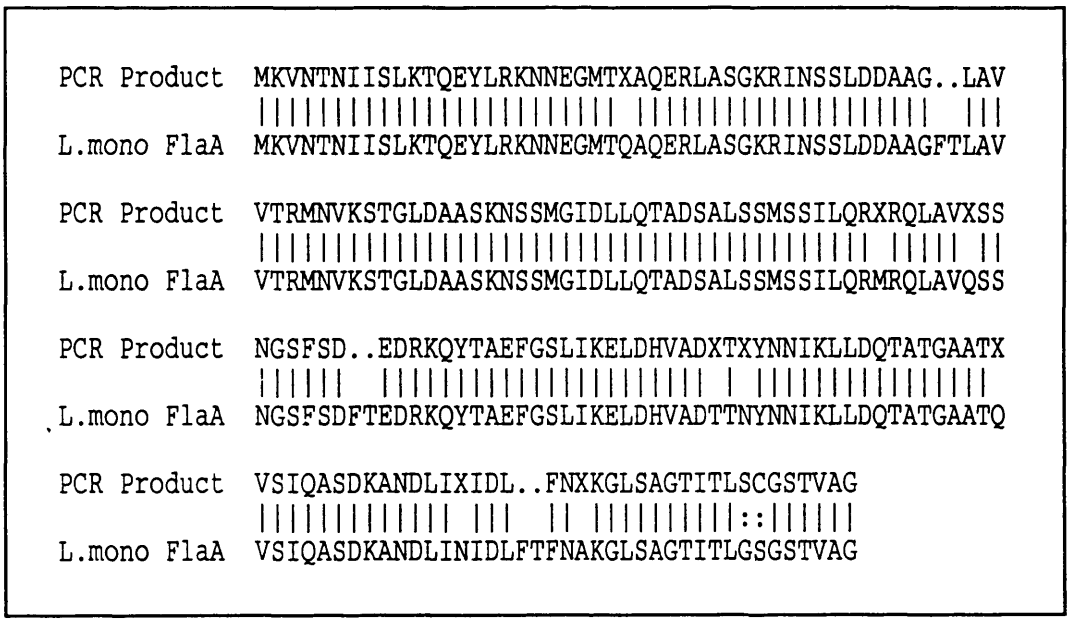

PCR Product MKVNTNIISLKTQEYLRKNNEGMTXAQERLASGKRINSSLDDAAG..LAV
L.mono FlaA MKVNTNIISLKTQEYLRKNNEGMTQAQERLASGKRINSSLDDAAGFTLAV
PCR Product VTRMNVKSTGLDAASKNSSMGIDLLQTADSALSSMSSILQRXRQLAVXSS
L.mono FlaA VTRMNVKSTGLDAASKNSSMGIDLLQTADSALSSMSSILQRMRQLAVQSS
PCR Product NGSFSD..EDRKQYTAEFGSLIKELDHVADXTXYNNIKLLDQTATGAATX
L.mono FlaA NGSFSDFTEDRKQYTAEFGSLIKELDHVADTTNYNNIKLLDQTATGAATQ
PCR Product VSIQASDKANDLIXIDL..FNXKGLSAGTITLSCGSTVAG
L.mono FlaA VSIQASDKANDLINIDLFTFNAKGLSAGTITLGSGSTVAG

Figure 3.34. Comparison of the deduced amino acid sequence of the insert in pJM9 with the listerial flagellin sequence. Comparison of the amino acid sequence deduced from the listerial DNA flanking the *erm*-distal end of the transposon, with the previously published FlaA sequence (accession number X65624). Amino acid residues are written in the single-letter code. Identical amino acids are indicated by a (|). Insertions included to obtain maximum homology are marked by a (.).

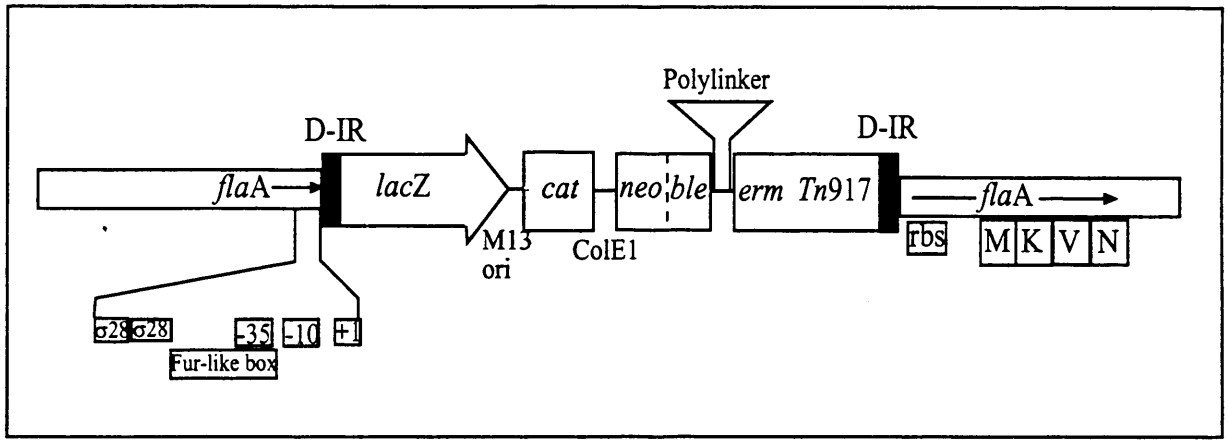

Polylinker
D-IR
D-IR
flaA
lacZ
cat
neo
ble
erm Tn917
flaA
M13
ori
ColE1
rbs
M
K
V
N
σ28
σ28
-35
-10
+1
Fur-like box

Figure 3.35. Diagrammatic representation of *Tn*917 insertion into the *flaA* gene of mutant 7D. Schematic diagram showing insertion of *Tn*917 into the *flaA* gene of *L. monocytogenes* mutant 7D. The chromosomal DNA at the *erm*-proximal side contains the promoter region of the gene (indicated by -10, -35, σ^{28}, and fur-like box) until the transcription initiation site (indicated by +1) while the chromosomal DNA at the *erm*-distal end of insertion contains the ribosome binding site (indicated by rbs) and the structural flagellin encoding sequence (indicated by M, K, V, N, the first four amino acids of FlaA [Dons *et al.*, 1992]). Details of the transposon were given previously. The diagram is not to scale.

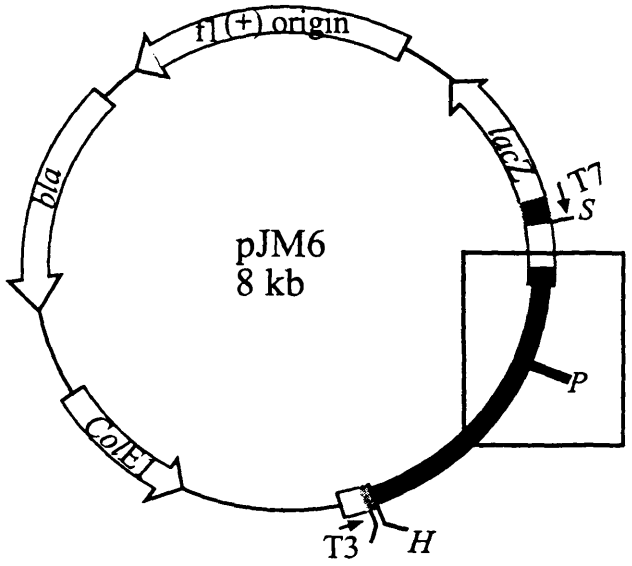
3.6.2 Analysis of the region of DNA in mutant 7D with significant homology to the *cheV* gene of *B. subtilus*

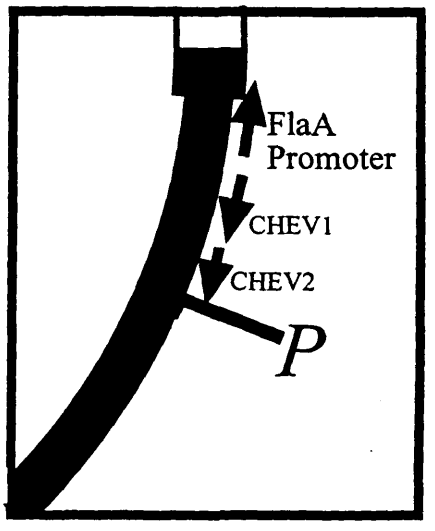
Although the objective of this section of the study was to determine the nucleotide sequence of the chromosomal DNA into which Tn*917* had inserted, the region upstream from the *flaA* promoter was analysed further, as the preliminary data shown in section 3.6 suggested the presence of a previously undescribed listerial gene in this region. This gene was shown to be 55% identical at the amino acid level to *cheV* of *B. subtilis.*

The preliminary data (Figure 3.28A) showed the presence of 290 bp of DNA, upstream from the *flaA* promoter, which encoded ORFA and was shown to have 55% amino acid identity to the C-terminal of the CheV protein of *B. subtilus* (Fredrick & Helmann, 1994). In this section the sequence of the remainder of this gene was determined and the encoding amino acid sequence was compared with CheV and and other Che proteins.

To obtain more sequence for this listerial CheV-like protein, plasmid pJM6, was used. A description of how this plasmid was constructed was given previously (Figure 3.24). pJM6 is an 8 kb plasmid containing 5 kb of listerial chromosomal DNA which flanks the *erm*-proximal end of the transposon. Complete sequence data was obtained in section 3.6 for the 500 bp immediately flanking the *erm*-proximal side of the transposon (see Figure 3.28A) and this included the region with homology to the *che*V of *B. subtilis.* Primers CHEV1 (TCGGCTTACTTACTTGTGCGTCGGC) and CHEV2 (GAAAATATGTTGCTTGGGCTTCGCG) were based on the region of pJM6 with homology to the *che*V gene of *B. subtilis.* They were synthesised in the sense direction to facilitate priming towards the unsequenced chromosomal DNA in pJM6. This is shown diagrammatically in Figure 3.36. A fragment of approximately 600 bp was successfully sequenced using primers CHEV1 and CHEV2 and the sequence is

Figure 3.36. Map of pJM6 indicating the positions of primers CHEV1 and CHEV2. Detailed map of pJM6, essentially pBluescript^{SK} containing a 5 kb *Hind*III-*Sal*I fragment of listerial DNA from the *erm*-proximal side of transposon insertion. The approximate locations of the CHEV1 and CHEV2 primers are indicated.


f1 (+) origin
lacZ
T7
S
bla
pJM6
8 kb
P
ColE1
T3
H


FlaA
Promoter
CHEV1
CHEV2
P

shown in Figure 3.37. The predicted amino acid sequence is shown below the nucleotide sequence. The sequence which was determined initially in section 3.6 (Figure 3.28A) is shown in double strand; the position of the CHEV1 and CHEV2 primer sites are also shown. Athough time limitation did not allow sequencing of both the sense and antisense strands of this fragment of DNA during this study, the nucleotide sequence and deduced amino acid sequence obtained, allowed comparisons to be made with sequences in databases using BLASTN AND BLASTX programs (National Centre for Biotechnology Information, Los Alamos, N.MEX).

When the amino acid sequence shown in Figure 3.37 was searched against protein database using the BLAST programs, homology was found to a number of proteins belonging to a family of proteins known as two component regulators. The highest similarity:identity (61%:55%), was to the CheV protein of *B. subtilis* (Fredrick & Helmann, 1994). An alignment of this novel listerial sequence with the *B. subtilis* CheV is shown in Figure 3.38. The *B. subtilis* CheV protein has been shown to contain two functional domains: the N-terminal domain showing similarity to the sensor protein CheW and the C-terminal domain showing similarity to the CheY response regulator domain and these are indicated in Figure 3.38. The active site residues (amino acid E181, D182 and K287) and the site of phosphorylation of this protein (amino acid D236) which have previously been described (Fredrick & Helmann. 1994) in the CheY-like domain of the *B. subtilis* CheV were also present in the *listerial* protein. The CheW-like domain of the B. subtilis protein is 90 amino acids longer than putative listerial CheW-like domain (Figure 3.38), however the significance of this was not investigated further.

The listerial CheV amino acid sequence determined in this study was aligned with the recently published sequence of the listerial CheY protein (Dons *et al.*, 1994) using Genetic Computer Group programs (Figure 3.39). This alignment confirmed the homology of the listerial CheV C-terminus to the response regulator CheY. In this

Figure 3.37. Nucleotide sequence and deduced amino acid sequence of a novel listerial gene. The nucleotide sequence and deduced amino acid sequence of a 800 bp fragment of listerial DNA which lies upstream from the *flaA* promoter region. The putative -10 and -35 regions are indicated. The stop codon and possible downstream termination sequences are also indicated. The sequence which was determined previously in section 3.6 (Figure 3.28A) is shown in double strand; the position of the CHEV1 AND CHEV2 primer sites are indicated.

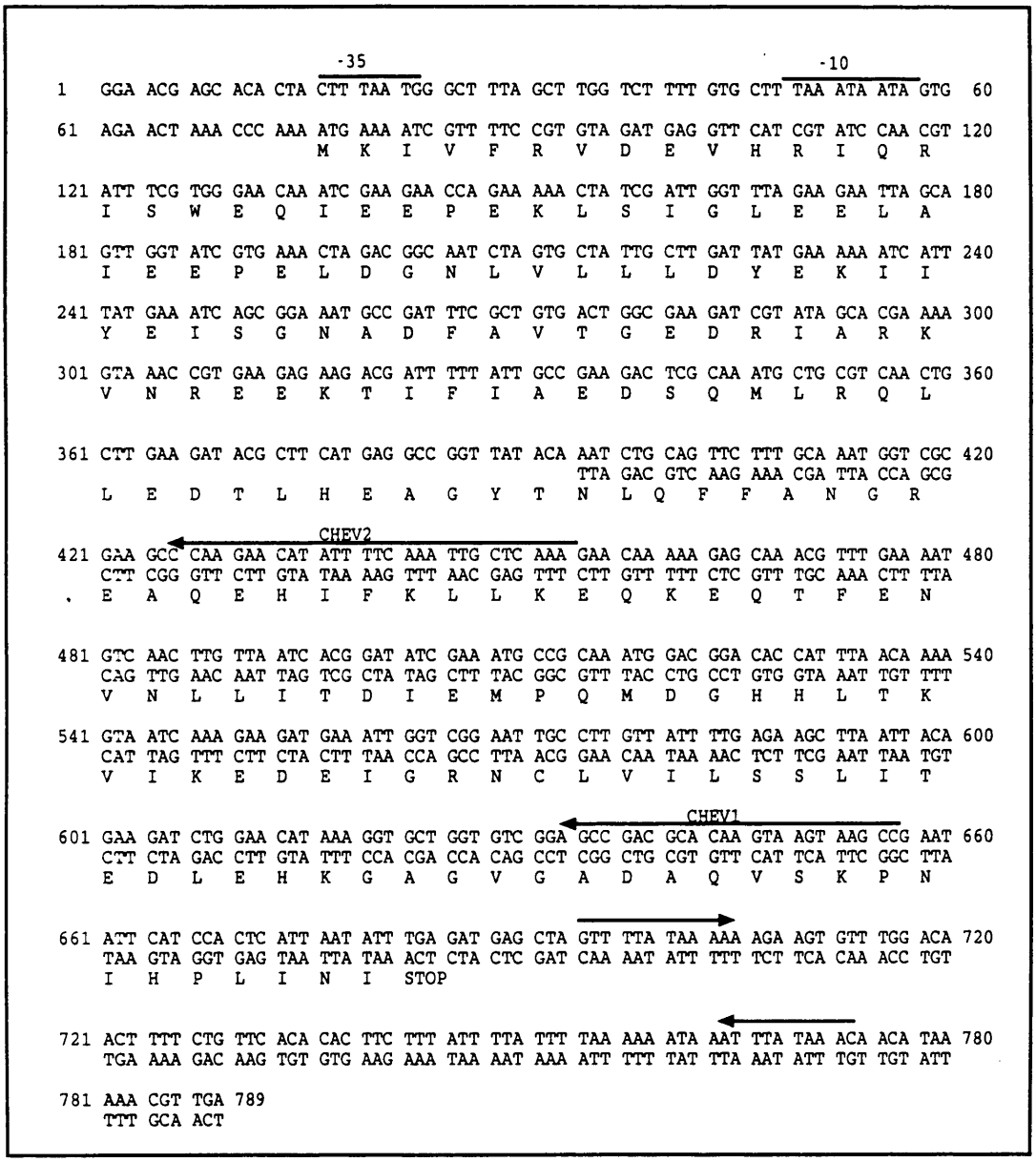

-35
-10
CHEV2
CHEV1

Figure 3.38. Amino acid comparison with CheV of *B. subtilis*. Comparison of the amino acid sequence from Figure 3.37 with the already published CheV sequence of *B subtilis* (Fredrick & Helmann, 1994; accession number: Z29584). Amino acid residues are written in the single-letter code. Identical amino acids are indicated by a (*) and conserved substitutions by (:). The two functional domains of CheV, with similarity to CheY and CheW of *B subtilis* are indicated. Conserved active site residues are indicated by (==) and (") and the site of phosphorylation is indicated by (+).

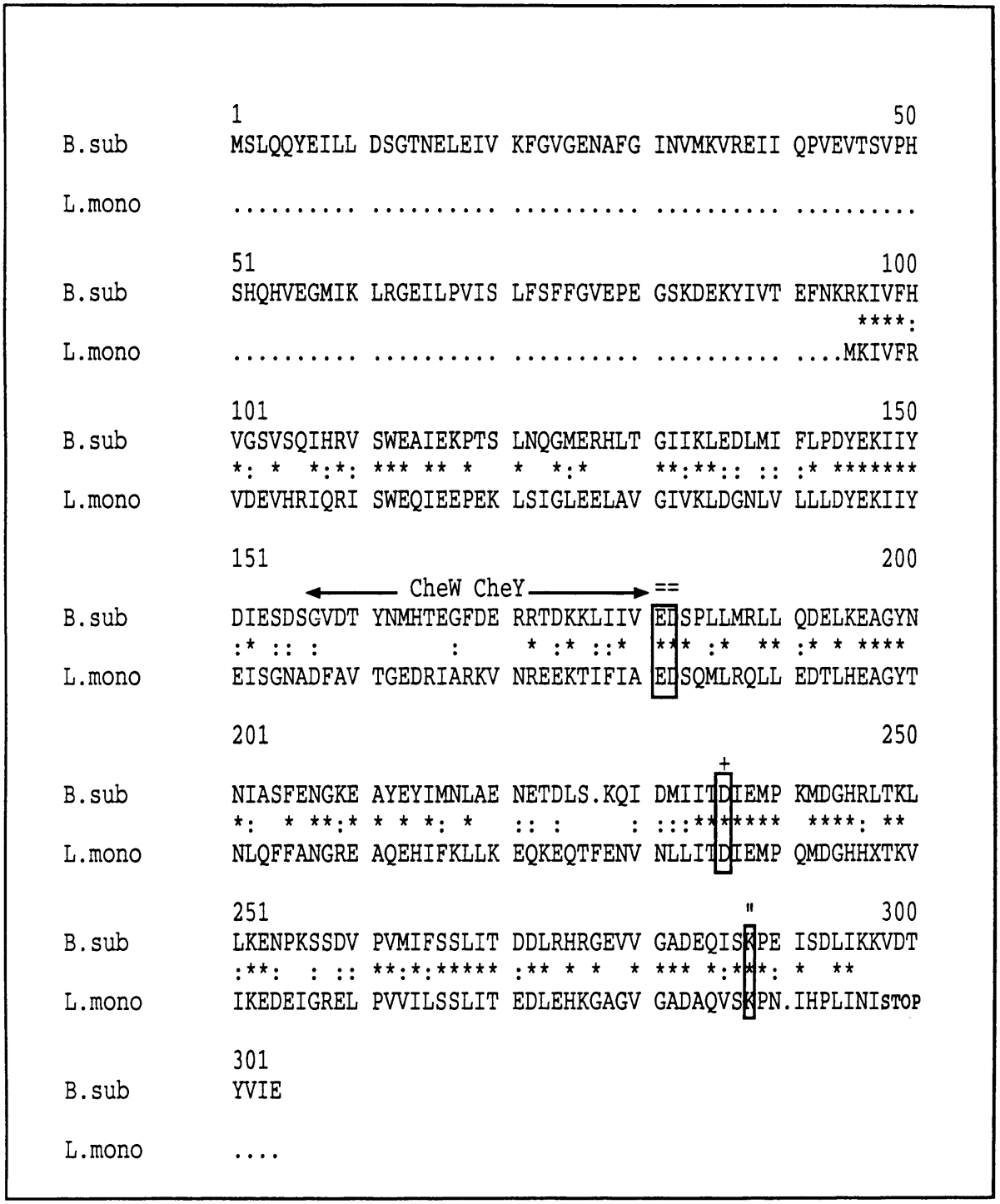

1 50
B.sub MSLQQYEILL DSGTNELEIV KFGVGENAFG INVMKVREII QPVEVTSVPH
L.mono ....
51 100
B.sub SHQHVEGMIK LRGEILPVIS LFSFFGVEPE GSKDEKYIVT EFNKRKIVFH
****:
L.mono ....MKIVFR
101 150
B.sub VGSVSQIHRV SWEAIEKPTS LNQGMERHLT GIIKLEDLMI FLPDYEKIIY
L.mono VDEVHRIQRI SWEQIEEPEK LSIGLEELAV GIVKLDGNLV LLLDYEKIIY
151 200
CheW CheY
==
B.sub DIESDSGVDT YNMHTEGFDE RRTDKKLIIV EDSPLLMRLL QDELKEAGYN
L.mono EISGNADFAV TGEDRIARKV NREEKTIFIA EDSQMLRQLL EDTLHEAGYT
201 250
+
B.sub NIASFENGKE AYEYIMNLAE NETDLS.KQI DMIITDIEMP KMDGHRLTKL
L.mono NLQFFANGRE AQEHIFKLLK EQKEQTFENV NLLITDIEMP QMDGHHXTKV
251 300
"
B.sub LKENPKSSDV PVMIFSSLIT DDLRHRGEVV GADEQISKPE ISDLIKKVDT
L.mono IKEDEIGREL PVVILSSLIT EDLEHKGAGV GADAQVSKPN.IHPLINISTOP
301
B.sub YVIE
L.mono ....

Figure 3.39. Alignment of this novel listerial protein with the listerial CheY protein. Alignment of the amino acid sequence from Figure 34 with the previously published CheY of *L. monocytogenes* (Dons *et al.*, 1994; accesion number X76170). Amino acid residues are written in the single-letter code. Identical amino acids are indicated by (*) and conserved substitutions by (:). Conserved active site residues are indicated by (==) and (") and the site of phosphorylation is indicated by (+).

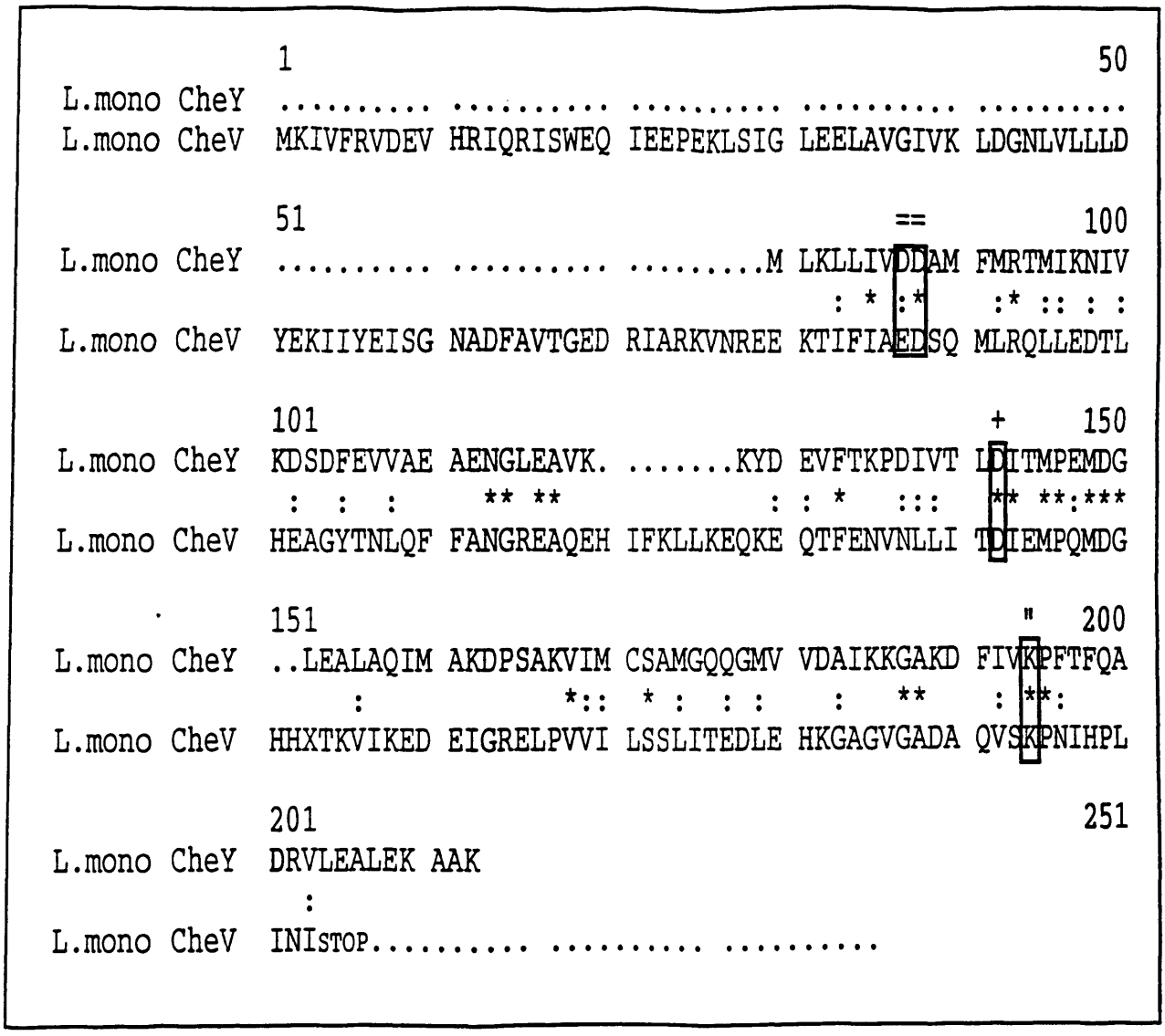

1 50
L.mono CheY ....
L.mono CheV MKIVFRVDEV HRIQRISWEQ IEEPEKLSIG LEELAVGIVK LDGNLVLLLD
51 100
==
L.mono CheY ....M LKLLIVDDAM FMRTMIKNIV
: * :* :* :: : :
L.mono CheV YEKIIYEISG NADFAVTGED RIARKVNREE KTIFIAEDSQ MLRQLLEDTL
101 150
+
L.mono CheY KDSDFEVVAE AENGLEAVK. .....KYD EVFTKPDIVT LDITMPEMDG
: : : ** ** : : * ::: ** **:***
L.mono CheV HEAGYTNLQF FANGREAQEH IFKLLKEQKE QTFENVNLLI TDIEMPQMDG
151 200
"
L.mono CheY ..LEALAQIM AKDPSAKVIM CSAMGQQGMV VDAIKKGAKD FIVKPFTFQA
: *:: * : : : ** : **:
L.mono CheV HHXTKVIKED EIGRELPVVI LSSLITEDLE HKGAGVGADA QVSKPNIHPL
201 251
L.mono CheY DRVLEALEK AAK
:
L.mono CheV INIstop..... ....

Figure the presence of an additional 75 amino acids upstream from the CheY-like domain of this listerial CheV protein is evident. Presuming, the listerial CheV is identical to the *B. subtilis* CheV, these extra amino acids would represent the CheW-like domain of the listerial CheV. From Figure 3.38, it is evident that the listerial CheW-like domain is approximately 90 amino acids less than the *B. subtilis* CheW-like domain. To date, no listerial CheW protein has been described, thus, no further analysis of the N-terminal 75 amino acids listerial CheV protein was performed.

3.7 Sequence analysis of chromosomal DNA flanking the *erm*-proximal end of the transposon in mutant 9E

Sequencing of the entire fragment of *L. monocytogenes* DNA present in pJM2 was performed using the Automated Prism DyeDeoxy Cycle Sequencing kit (Applied Biosystems). Plasmid pJM2 is a 14 kb plasmid which was rescued from mutant 9E as described previously (section 3.5). It contained approximately 500 bp of chromosomal DNA which immediately flanked the *erm*-proximal side of the transposon. The primers used were designated ERM.PROX and HI-5 and the positions of the complimentary sites on pJM2 are shown in Figure 3.40. The ERM.PROX primer, GCAATAACCGTTACCTGTTTGTGCC, was based on a region of *Tn*917 120 bp from the *erm*-proximal inverse repeat (P-IR) (Shaw & Clewell, 1985) and was made in the antisense direction to facilitate priming towards the flanking chromosomal DNA. Primer HI-5, ACGAAAATAACTAAACTGCTTGGC, was based on a region of *Tn*917, 30 bp from the *Hind*III site at residue 3025 of *Tn*917 (Shaw & Clewell, 1985). This primer was made in the sense direction to enable sequencing of the chromosomal DNA insert in pJM2 (Figure 3.40). Sequencing of this chromosomal DNA insert in pJM2 was performed three times and the nucleotide sequence obtained is shown in Figure 3.41. The region from P-IR to the HindIII site in pJM2 is shown.

The sequence was searched against nucleic acid and amino acid databases using BLASTN and BLASTX programs (National Centre for Biotechnology Information, Los Alamos, N.MEX), available on the internet (http//:www.ncbi.nlm.nih.gov/cgi-bin/BLAST/). The results indicated that the listerial DNA fragment (approximately 320 bp) immediately flanking the *erm*-proximal end of the transposon was significantly homologous to a gene involved in the synthesis of an enzyme involved in bacterial arginine biosynthesis, *argC* (Cunin *et al.*, 1986). This gene has been found in *B. subtilis* (Smith *et al.*, 1990) and also in other bacteria including *Streptococcus mutans* (Cardineau & Curtiss, 1987), *E.coli* (Parsot *et al.*, 1988) and *Mycobacterium*

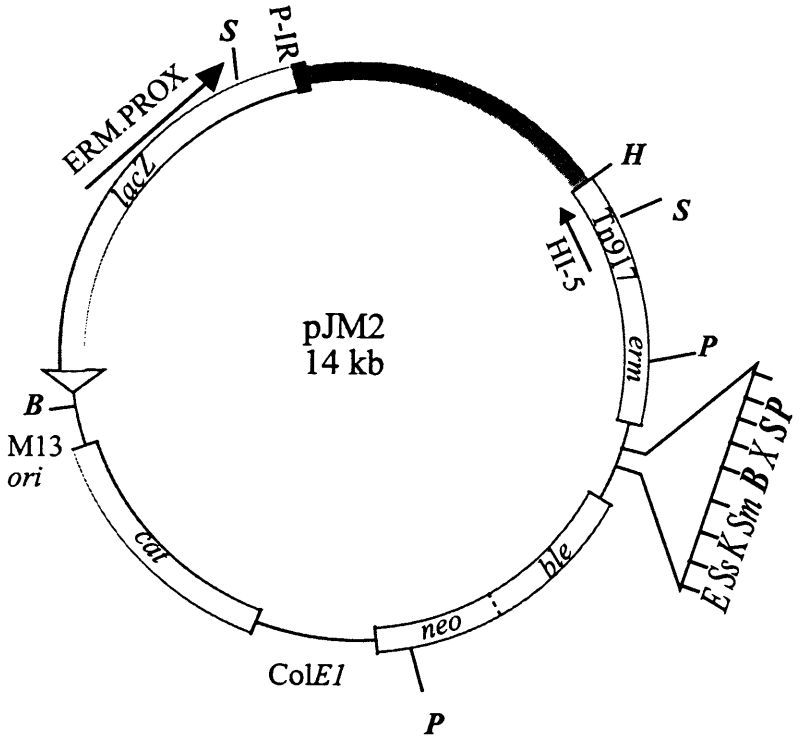

pJM2
14 kb
S
P-IR
ERM.PROX
lacZ
H
S
Tn917
HI-5
ere
P
B
M13
ori
cat
neo
ble
ColE1
P
E Ss K Sm B X Sp

Figure 3.40. Diagrammatic representation of pJM2 showing the ERM.PROX and HI-5 primer sites. Detailed map of plasmid pJM2 recovered from mutant 9E by *Hind*III digestion of chromosomal DNA, self ligation and transformation into *E. coli* MC1061. The positions and directions of the ERM.PROX and the HI-5 primers are shown.

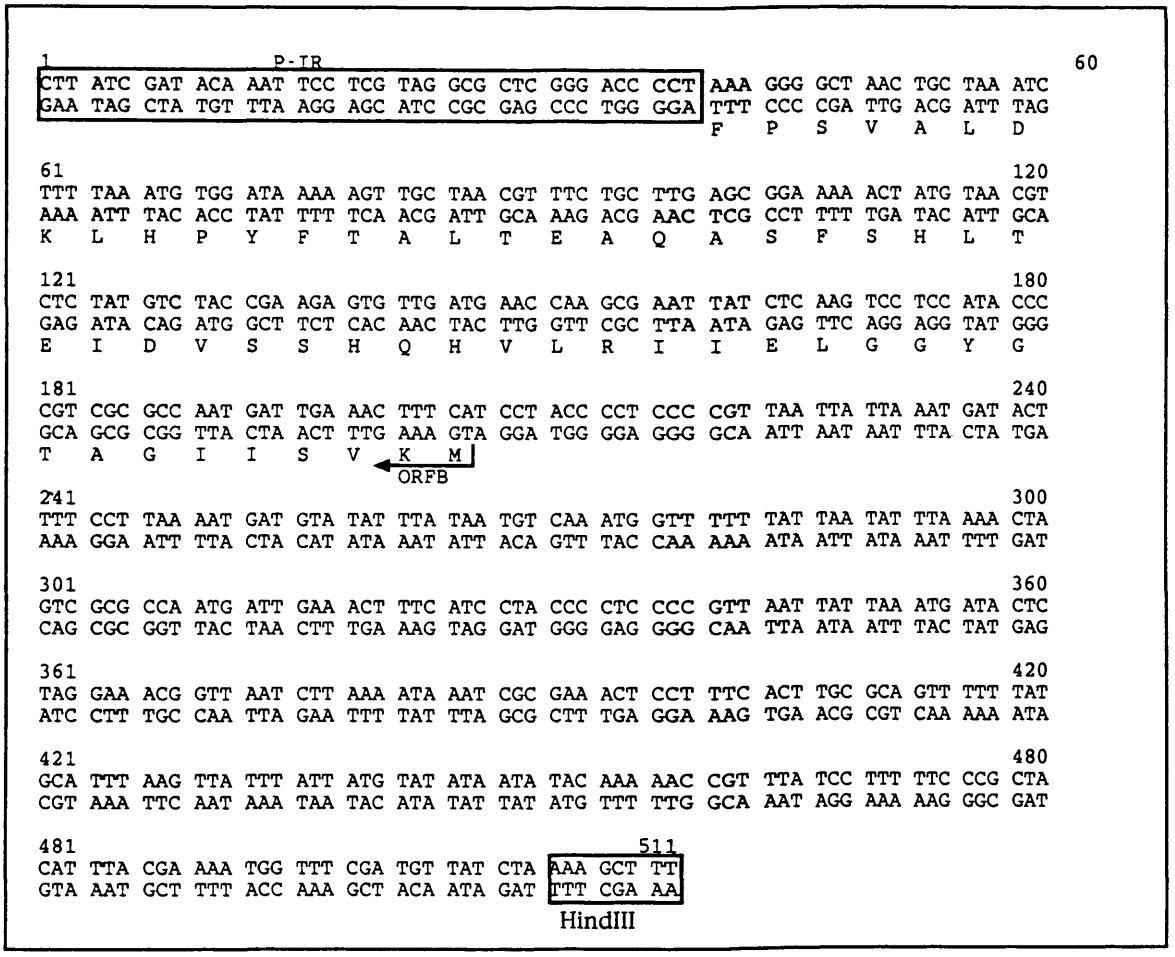

P-IR
ORFB
HindIII

Figure 3.41. Nucleotide sequence of the listerial DNA in pJM2. The sequence of the listerial DNA, from the *erm*-proximal inverse repeat (P-IR) to the *HindIII* site indicating the end of the listerial DNA, is shown. The deduced amino acid of ORFB is shown underneath the nucleotide sequence where the single letter code was used. Further detail on promoter region upstream from ORFB is given in Figure 3.43.

tuberculosis (Phillip *et al.*, 1996) and its role in arginine biosynthesis is discussed in more detail in section 4.4. The remaining sequence showed no similarity to any sequences in the database and was not analysed further.

The region with homology to *arg*C genes of other bacterial species was designated ORFB and is shown in Figure 3.41. An alignment of the deduced amino acid sequence of ORFB with ArgC proteins from other species is shown in Figure 3.42 and further details of the homologies are given in Table 3.6. As evident from Figures 3.41, 3.42 and the data in Table 3.6, ORFB is homologous to the N-terminus of the ArgC proteins and the orientation of the gene was towards the transposon, suggesting that the remaining gene sequence should be present at the *erm*-distal end of the transposon. This was investigated further (see below, section 3.7.1).

Not surprising is the fact that the region immediately upstream from ORFB in the *erm*-proximal chromosomal DNA was shown to be homologous to the *B. subtilis argC* promoter region (Smith *et al.*, 1986). This is shown in Figure 3.43. The positions of the putative -10 and -35 regions are indicated. Using the Genetic Computer Group programs attempts were made to identify an arginine box upstream from ORFB, the putative listerial *arg*C gene. Arginine boxes have been shown to exist upstream from a number of genes involved in the biosynthesis of arginine in *E. coli* (Parsot *et al.*, 1988) and *B. subtilis* (Smith *et al.*, 1990) and are involved in the regulated expression of *arg* genes by environmental arginine. A potential arginine box was identified upstream from ORFB and is shown in Figure 3.43.

Interestingly, in this study, the presence of a Fur-like box, similar to those identified upstream from iron-regulated genes of *E. coli* (de Lorenzo *et al.*, 1988) and other bacterial species (Tai & Holmes, 1988) was iedntified and is shown in Figure 3.43 also. This 18-bp motif was identified using Genetic Computer Group programs, where attempts were being made to identify a Fur-like box (de Lorenzo *et al.*, 1988) or

Figure 3.42. Comparison of the amino acid sequence of ORF B with the amino acid sequence of various ArgC proteins. Alignment of the ORF B from *L. monocytogenes* with ArgC proteins from other bacterial species, including *B. subtilis*, *E. coli*, *S. mutans* and *Mycobacterium tuberculosis*.

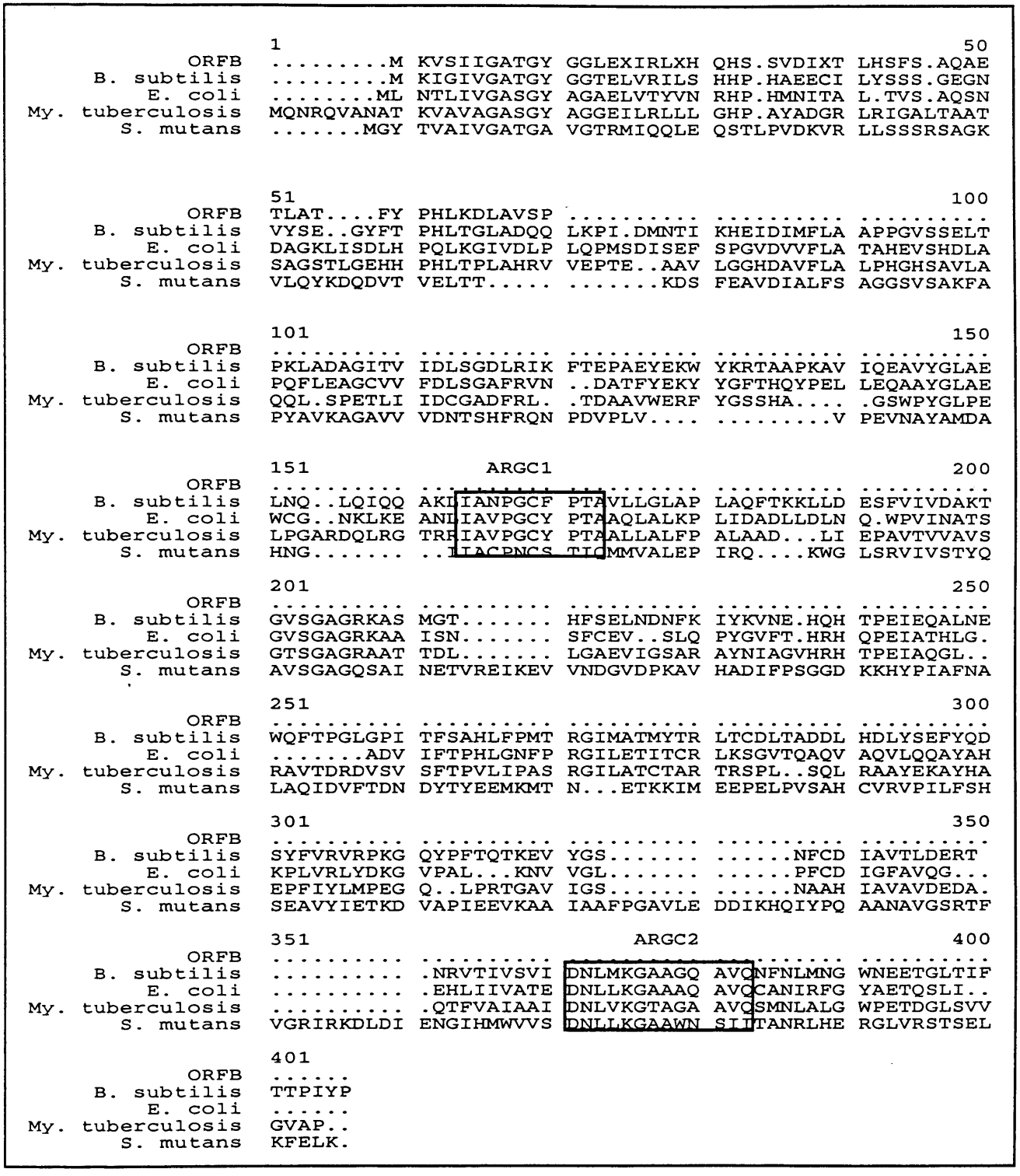

1 50
ORFB .....M KVSIIGATGY GGLEXIRLXH QHS.SVDIXT LHSFS.AQAE
B. subtilis .....M KIGIVGATGY GGTELVRILS HHP.HAEECI LYSSS.GEGN
E. coli .....ML NTLIVGASGY AGAELVTYVN RHP.HMNITA L.TVS.AQSN
My. tuberculosis MQNRQVANAT KVAVAGASGY AGGEILRLLL GHP.AYADGR LRIGALTAAT
S. mutans .....MGY TVAIVGATGA VGTRMIQQLE QSTLPVDKVR LLSSSRSAGK
51 100
ORFB TLAT....FY PHLKDLAVSP ....
B. subtilis VYSE..GYFT PHLTGLADQQ LKPI.DMNTI KHEIDIMFLA APPGVSSELT
E. coli DAGKLISDLH PQLKGIVDLP LQPMSDISEF SPGVDVVFLA TAHEVSHDLA
My. tuberculosis SAGSTLGEHH PHLTPLAHRV VEPTE..AAV LGGHDAVFLA LPHGHSAVLA
S. mutans VLQYKDQDVT VELTT..... .....KDS FEAVDIALFS AGGSVSAKFA
101 150
ORFB ....
B. subtilis PKLADAGITV IDLSGDLRIK FTEPAEYEKW YKRTAAPKAV IQEAVYGLAE
E. coli PQFLEAGCVV FDLSGAFRVN ..DATFYEKY YGFTHQYPEL LEQAAYGLAE
My. tuberculosis QQL.SPETLI IDCGADFRL. .TDAAVWERF YGSSHA.... .GSWPYGLPE
S. mutans PYAVKAGAVV VDNTSHFRQN PDVPLV.... .....V PEVNAYAMDA
151 ARGC1 200
ORFB ....
B. subtilis LNQ..LQIQQ AKIIANPGCF PTAVLLGLAP LAQFTKKLLD ESFVIVDAKT
E. coli WCG..NKLKE ANIIAVPGCY PTAAQLALKP LIDADLLDLN Q.WPVINATS
My. tuberculosis LPGARDQLRG TRIIAVPGCY PTAALLALFP ALAAD...LI EPAVTVVAVS
S. mutans HNG..... ..IIACPNCS TIQMMVALEP IRQ....KWG LSRVIVSTYQ
201 250
ORFB ....
B. subtilis GVSGAGRKAS MGT..... HFSELNDNFK IYKVNE.HQH TPEIEQALNE
E. coli GVSGAGRKAA ISN..... SFCEV..SLQ PYGVFT.HRH QPEIATHLG.
My. tuberculosis GTSGAGRAAT TDL..... LGAEVIGSAR AYNIAGVHRH TPEIAQGL..
S. mutans AVSGAGQSAI NETVREIKEV VNDGVDPKAV HADIFPSGGD KKHYPIAFNA
251 300
ORFB ....
B. subtilis WQFTPGLGPI TFSAHLFPMT RGIMATMYTR LTCDLTADDL HDLYSEFYQD
E. coli .....ADV IFTPHLGNFP RGILETITCR LKSGVTQAQV AQVLQQAYAH
My. tuberculosis RAVTDRDVSV SFTPVLIPAS RGILATCTAR TRSPL..SQL RAAYEKAYHA
S. mutans LAQIDVFTDN DYTYEEMKMT N...ETKKIM EEPELPVSAH CVRVPILFSH
301 350
ORFB ....
B. subtilis SYFVRVRPKG QYPFTQTKEV YGS..... .....NFCD IAVTLDERT
E. coli KPLVRLYDKG VPAL...KNV VGL..... .....PFCD IGFAVQG...
My. tuberculosis EPFIYLMPEG Q..LPRTGAV IGS..... .....NAAH IAVAVDEDA.
S. mutans SEAVYIETKD VAPIEEVKAA IAAFPGAVLE DDIKHQIYPQ AANAVGSRTF
351 ARGC2 400
ORFB ....
B. subtilis ..... .NRVTIVSVI DNLMKGAAGQ AVQNFNLMNG WNEETGLTIF
E. coli ..... .EHLIIVATE DNLLKGAAAQ AVQCANIRFG YAETQSLI..
My. tuberculosis ..... .QTFVAIAAI DNLVKGTAGA AVQSMNLALG WPETDGLSVV
S. mutans VGRIRKDLDI ENGIHMWVVS DNLLKGAAWN SIITANRLHE RGLVRSTSEL
401
ORFB ......
B. subtilis TTPIYP
E. coli ......
My. tuberculosis GVAP..
S. mutans KFELK.

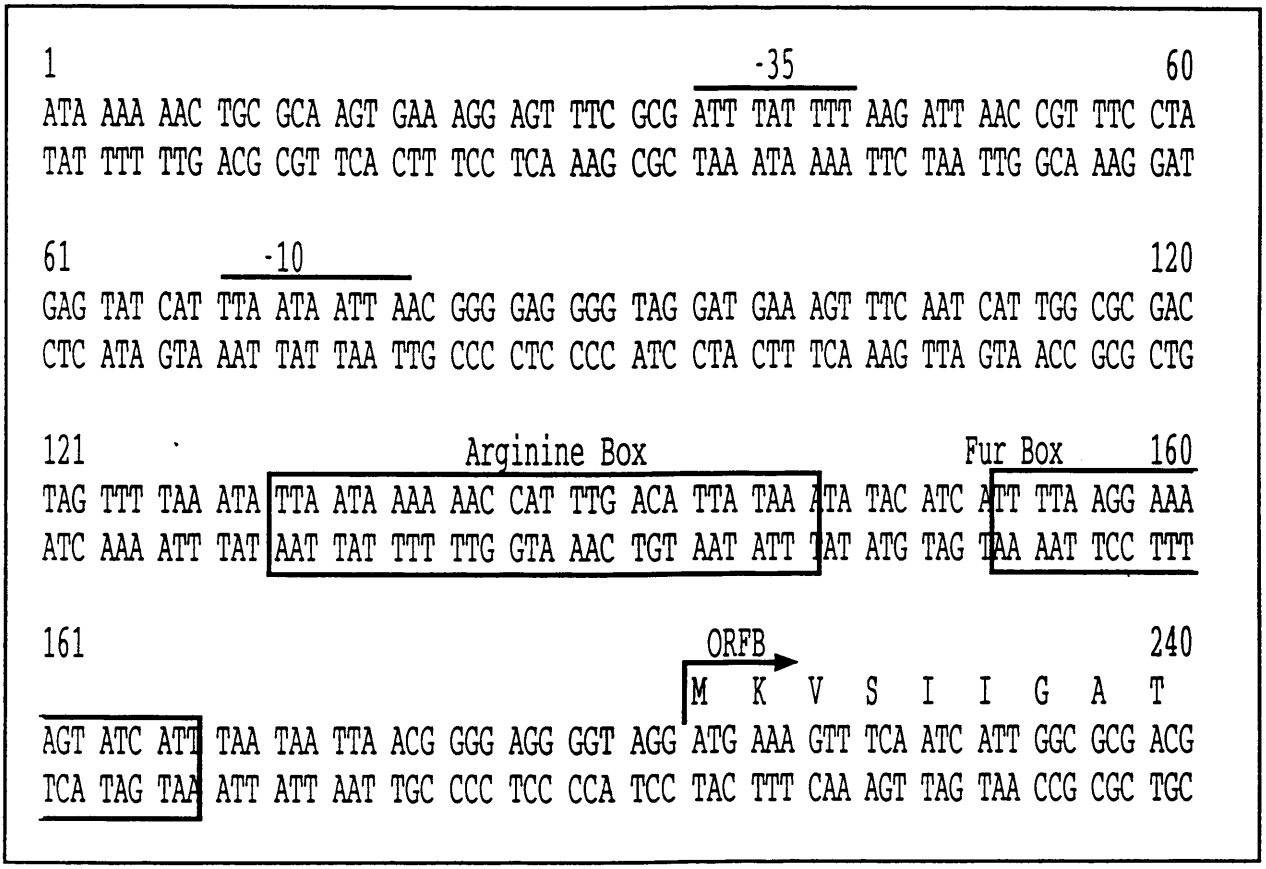
Table 3.6. Details of homologies observed between ORFB from *L. monocytogenes* and ArgC proteins of other bacterial species.

Homology[1]	Organism	Protein[2] (Region)	Accession Number	Reference
42% I 57% S	*B. subtilis*	ArgC (a.a. 1-52)	M15420	Smith *et al.*, (1990)
37% I 62% S	*E. coli*	ArgC (a.a. 6-32)	M21446	Parsot *et al.*, (1988)
38% I 45% S	*S. mutans*	ArgC (a.a. 4-49)	P10539	Cardineau &Curtiss, (1987)
50% I 68% S	My. tuberculosis	ArgC (a.a. 11-32)	Z85982	Phillip *et al.*, (1996)

[1]I=identity; S=similarity

[2]region of ArgC with homology to ORFB; a.a.=amino acid

Figure 3.43. Comparison of the promoter region from the *B. subtilis argC* gene with a region upstream from ORFB in mutant 9E. The region from residues 282 to 346 in Figure 38 was shown to have 70 % similarity and 67 % identity to the region from residues 22 to 87 of the *B. subtilis argC* nucleotide sequence. This region represents the *argC* promoter region in *B. subtilis*. The positions of the putative Fur box and Arginine box are shown in addition to the the translational start codon for ORFB.


1
-35
60
ATA AAA AAC TGC GCA AGT GAA AGG AGT TTC GCG ATT TAT TTT AAG ATT AAC CGT TTC CTA
TAT TTT TTG ACG CGT TCA CTT TCC TCA AAG CGC TAA ATA AAA TTC TAA TTG GCA AAG GAT
61
-10
120
GAG TAT CAT TTA ATA ATT AAC GGG GAG GGG TAG GAT GAA AGT TTC AAT CAT TGG CGC GAC
CTC ATA GTA AAT TAT TAA TTG CCC CTC CCC ATC CTA CTT TCA AAG TTA GTA ACC GCG CTG
121
Arginine Box
Fur Box
160
TAG TTT TAA ATA TTA ATA AAA AAC CAT TTG ACA TTA TAA ATA TAC ATC ATT TTA AGG AAA
ATC AAA ATT TAT AAT TAT TTT TTG GTA AAC TGT AAT ATT TAT ATG TAG TAA AAT TCC TTT
161
ORFB
240
M K V S I I G A T
AGT ATC ATT TAA TAA TTA ACG GGG AGG GGT AGG ATG AAA GTT TCA ATC ATT GGC GCG ACG
TCA TAG TAA ATT ATT AAT TGC CCC TCC CCA TCC TAC TTT CAA AGT TAG TAA CCG CGC TGC

a Dtx-recognition sequence (Murphy *et al.*, 1978; Tai & Homes, 1988) in the *listerial* DNA sequence. As mentioned previously in section 3.6, the reason behind looking for these motifs was due to the iron-related phenotype of mutant 7D. The Fur-like box is typically found upstream from iron-regulated genes of Gram-negative species, although a similar motif was found upstream from the *listerial kat*G gene (Haas, *et al.*, 1991). The Dtx recognition sequence was identified upstream from the *Corynebacterium diptheriae*, iron-regulated, *tox* gene and was thought to be a Gram-positive equivalent of the Fur box (Murphy, *et al.*, 1978). This finding of a Fur-like box upstream from *arg*C is discussed in more detail in section 4.4. Thus, having identified the N-terminal region of a putative *arg*C gene at the *erm*-proximal end of transposition, the next stage of the investigation was to determine the sequence of the chromosomal DNA flanking the *erm*-distal end of transposition to confirm the presence of the remainder of *arg*C.

3.7.1 PCR amplification and sequencing of chromosomal DNA at the erm-distal end of the transposon in mutant 9E

To confirm that the transposon had inserted into a putative *argC* gene of *L. monocytogenes*, the chromosomal DNA flanking the *erm*-distal end of the transposon was sequenced. This involved a similar approach to that adopted for cloning and sequencing the *erm*-distal DNA from mutant 7D (section 3.6.1). A fragment of DNA containing part of the *erm*-distal end of the transposon and a region of chromosomal DNA immediately flanking this was amplified by PCR. Primers ERM.DIST GGCCTTGAAACATTGGTTTAGTGGG, based on a region of *Tn*917, 100 bp from the *erm*-distal inverse repeat (D-IR) (Shaw & Clewell, 1985) was used again while the other primers used where designated ARGC1 and ARGC2. Primers ARGC1, GCCTGGATTGGCAATGAGTTTCGC, and ARGC2, TAAAGTTTTGCACTGCCTGACCGGC, were based on regions of the *B. subtilus argC* gene (Smith *et al.*, 1990;) and are indicated previously in Figure 3.42. Primers

were based on regions of conservation among proteins encoded by the *argC* genes of different bacteria. An alignment of the nucleotide sequences of the argC genes from *B. subtilis*, *E. coli*, *Streptococcus mutans* and *Mycobacterium tuberculosis*, showing the positions of the ARGC1 and ARGC2 primer sites is shown in Figure 3.44. Primers ARGC1 and ARGC2 were made in the antisense directions to facilitate priming towards the transposon. A simplified diagram of the approach to sequence the *erm*-distal flanking DNA is shown in Figure 3.45 and the presumed positions of the ARGC1 and ARGC2 primer sites are indicated.

From the known sequence of *Tn*917 (Shaw & Clewell, 1985), it was predicted that the ERM.DIST primer site is approximately 50 bp from the distal inverse repeat (D-IR). From the known sequence of *argC* from *B. subtilis* in addition to the known sequence of the listerial chromosomal dNA flanking*erm*-proximal flanking chromosomal DNA, the ARGC1 and ARGC2 primer sites were predicted to be 300 bp and 750 bp from the D-IR, respectively. The predicted length of PCR products were 400 bp and 850 kb from ERM.DIST to ARGC1 and ERM.DIST to ARGC2, respectively.

PCR reactions were performed as described previously, using Taq DNA polymerase and 35 cycles of: 94°C, 30 sec; 60°C , 30 sec; 72°C, 3 min. The resulting PCR products were analysed on a 0.7% (w/v) agarose gel (Figure 3.46). Lane 2 shows the PCR product obtained when chromosomal DNA from mutant 9E was used as the template and ERM.DIST and ARGC2 were used as primers. A band of approximately 850 bp was visible. No PCR products were produced when DNA from mutant 9E and primers ERM.DIST and ARGC1 were used (Lane 1) or when DNA from the wild type strain was used as the template with primers ERM.DIST and ARGC2 (Lane 3). No product was expected for the latter reaction, however, a product of approximately 350 bp was expected from the former reaction. The reason why no product was produced using the ARGC1 and the ERM.DIST primers was not investigated further.

```
                1                                                   50
     B. subtilis  ......... ......... ......... ..TTGAAAAT AGGAATTGTA
         E. coli  ......... ......... ......... TGTTGAATAC GCTGATTGTG
 My. tuberculosis ......... ......... ......... ......... .........
        S. mutans AATGAATTAG AATAAAGAGG TAAACTATGG GCTACACAGT TGCTATCGTT

               51                                                  100
     B. subtilis GGTGCTACAG GATATGGAGG CACCGAACTT GTCAGGATTC TTTCGCATCA
         E. coli GGTGCCAGCG GCTACGCTGG CGCAGAGCTA GTGACCTATG TAAATCGCCA
 My. tuberculosis ......... ......... ......... ......... .........
        S. mutans GGTGCTACAG GCGCCGTTGG AACTCGTATG ATTCAACAAT TGGAACAATC

              101                                                  150
     B. subtilis TCCTCATGCA GAGGAATGCA TACTTTATTC ATC....... ..CAGCGGAG
         E. coli TCCGCATATG AACATAACCG CTTTGACTGT TTCAGCGCAA AGCAATGATG
 My. tuberculosis ......... ......... ......... ......... .........
        S. mutans GACACTTCCA GTTGATAAGG TACGGCTTTT GTCATCTTCA CGTTCTGCAG

              151                                                  200
     B. subtilis AAGGGAATGT CTATAGCGAG GGTTA..... .........TC CTCATCTTAC
         E. coli CGGGAAAGTT AATCTCCGAT TTGCA..... .........TC CGCAGCTAAA
 My. tuberculosis .........C GCTCGGCGAA CACCA..... .........TC CGCACCTGAC
        S. mutans GTAAAGTTTT GCAATATAAA GATCAAGATG TCACGGTTGA ATTAACTACG

              201                                                  250
     B. subtilis CGGCTTAGCG GATCAGCAGC TGAAGCCGAT ...TGATATG AATACGATCA
         E. coli AGGCATCGTT GATCTGCCGT TGCAGCCGAT GTCGGATATC AGCGAGTTTA
 My. tuberculosis GCCGCTGGCC CATCGAGTAG TCGAACCCAC CGAAGCT... ...GCCGTGC
        S. mutans AAAGATTCCT TTGAAGCTGT TGATATTGCG CTTTTTTCAG CTGGCGGTTC

              251                                                  300
     B. subtilis AACACGAAAT AGATATCATG TTTCTCGCTG CGCCGCCCGG AGTATCAAGT
         E. coli GCCCAGGGGT GGACGTAGTG TTTCTCGCCA CCGCCCATGA AGTTAGCCAC
 My. tuberculosis TCGGTGGCCA TGACGCCGTC TTCTTGGCCT TGCCGCACGG GCATTCGGCG
        S. mutans TGTTTCGGCA AAATTTGCTC CCTATGCAGT CAAAGCTGGT GCAGTCGTTG

              301                                                  350
     B. subtilis GAATTGACTC CAAAGCTGGC AGACGCGGGA ATTACGGTTA TTGATCTGTC
         E. coli GATTTAGCGC CGCAGTTTCT TGAAGCGGGC TGCGTGGTGT TCGACCTTTC
 My. tuberculosis GTGTTGGCGC AGCAACTG.. .AGCCCCGAG ACACTGATCA TCGACTGCGG
        S. mutans TTGATAATAC CTCTCATTTT CGTCAAAATC CAGATGTGCC TTTGGTTGTT

              351                                                  400
     B. subtilis AGGTGATCTG AGGATAAAAG AACCGGCTGA ATATGAAAAA TGGTATAAAC
         E. coli CGGCGCGTTT CGTGTTAACG ACGCCACCTT CTATGAAAAA TATTACGGCT
 My. tuberculosis GGCGGACTTT CGGCTCACCG ACGCCGCCGT CTGGGAGCGG TTCTACGGGT
        S. mutans CCTGAAGTCA ATGCTTATGC TATGGATGCT CATAATGGGA TTATTGCTTG

              401                                                  450
     B. subtilis GGACAGCGGC ..ACCGAAGG CGGTGATTCA AGAGGCGGTA TACGGTCTGG
         E. coli TTACCCATCA ..ATACCCGG AACTGTTGGA ACAGGCAGCC TACGGTCTGG
 My. tuberculosis CGTCGCACGC CGGTAGCTGG CCGTATGGGT TGCCCGAGCT GCCGGGCGCG
        S. mutans TCCTAACTGC TCAACGATTC AAATGATGGT AGCCTTGGAA CCTATTCGTC

              451                                  <<<<ARGC1<<<< 500
     B. subtilis CAGAACTGAA TCAACTGCAA ATTCAACAGG CGAAACTCAT TGCCAATCCA
         E. coli CGGAGTGGTG CGGTAATAAA TTAAAAGAAG CGAATTTGAT TGCGGTGCCG
 My. tuberculosis CGGGAC.... .......CAA TTGCGCGGCA CCCGCCGCAT CGCGGTGCCC
        S. mutans AAAAATGGGG ATTAAGTCGT GTTATTGTTT CAACCTATCA AGCTGTTTCA

              501                                                  550
     B. subtilis GGCTGTTTTC CAACAGCTGT TTTGCTTGGC CTCGCGCCAT TGGCTCAAAA
         E. coli GGCTGTTATC CGACGGCGGC ACAGCTGGCG CTGAAACCGT TGATTGATGC
 My. tuberculosis GGCTGCTATC CGACCGCGGC ACTGCTGGCG CTTTTTCCCG CGCTGGCCGC
        S. mutans GGAGCAGGTC AATCAGCTAT TAATGAAACT GTTCGTGAAA TTAAAGAAGT
```

Figure 3.44. Alignment of *argC* nucleic acid sequences from various bacterial speices: postions of ARGC1 and ARGC2 primer sites. The nucleotide sequences of argC genes from *B. subtilis* (Smith *et al.*, 1990; M15420), *E. coli* (Parsot *et al.*, 1988; M21446), *S. mutans* (Cardineau & Curtiss, 1987; P1053) and *M. tuberculosis* (Phillip *et al.*, 1996; Z85982) were aligned and the regions on which the ARGC1 and ARGC2 primers were based was highlighted.

```
                551                                                   600
     B. subtilis GAAACTGCTC GA...TGAAT CTTTCGTTAT CGTTGACGCG AAGACCGGTG
         E. coli CGATCTTCTT GACCTCAATC AGTGGCCGGT GATCAACGCC ACCAGCGGCG
My. tuberculosis AGACCTTATC GAGCCCG... ...CGGTGAC CGTGGTCGCC GTGAGCGGTA
       S. mutans TGTTAATGAT GGTGTGGATC CTAAAGCTGT TCATGCTGAT ATTTTTCCAT

                601                                                   650
     B. subtilis TTTCCGGAGC GGGAAGAAAA GCATCCATGG GAACTCATTT TTCTGAGCTG
         E. coli TGAGCGGTGC AGGGCGTAAA GCGGCCATTT CAAACAGCTT TTGTGAAGTT
My. tuberculosis CCTCGGGGGC GGGTCGTGCG GCCACCACCG ACTTGCTGGG CGCGGAGGTC
       S. mutans CAGGTGGTGA TAAAAAGCAT TATCCAATTG CTTTCAATGC TTTGGCACAG

                651                                                   700
     B. subtilis AACGACAATT TTAAAATTTA TAA...AGTC AATGAACATC AGCACACGCC
         E. coli AGC......C TGCAACCGTA TGG...CGTC TTTACTCATC GCCATCAACC
My. tuberculosis ATCGGGTCGG CGCGCGCCTA CAACATCGCC GGCGTCCACC GGCACACCCC
       S. mutans ATTGATGTCT TCACCGATAA TGATTATACT TATGAAGAAA TGAAGATGAC

                701                                                   750
     B. subtilis GGAAATTGAG CAGGCGCTGA ATGAATGGCA GCCAGGTCTC GGGCCCATTA
         E. coli AGAGATCGCC ACACACCTCG GT..... ..... GCTGACGTTA
My. tuberculosis CGAGATCGCT CAAGGGCTAC GCGCGGTCAC CGACCGCGAC GTCTCGGTCT
       S. mutans TAACGAAACC AAGAAAATCA TGGAAGAACC TGAACTTCCC GTTTCGGCCC

                751                                                   800
     B. subtilis CATTTTCGGC TCACTTGTTT CCGATGACAA GGGGCATCAT GGCGACGATG
         E. coli TCTTCACCCC ACATCTGGGC AATTTCCCGC GCGGCATTCT CGAAACCATT
My. tuberculosis CGTTTACCCC GGTGCTGATC CCGGCCTCCC GTGGCATCCT GGCCACCTGC
       S. mutans ATTGTGTTCG TGTTCCAATC CTTTTTTCA. ....CATTCT GAGGCTGTTT

                801                                                   850
     B. subtilis TATACCAGAT TAACCTGTGA CCTAACCGCA GATGACCTGC ATGATTTATA
         E. coli ACCTGCCGCC TGAAATCGGG TGTGACCCAG GCGCAAGTCG CGCAAGTGTT
My. tuberculosis ACGGCACGCA CCCGATC... ...ACCCCTG TCGCAGCTGC GGGCAGCCTA
       S. mutans ATATTGAAAC TAAAGACGTT GCTCCAATTG AAGAAGTAAA AGCAGCTATT

                851                                                   900
     B. subtilis TTCGGAATTT TACCAAGATT CATATTTTGT GAGAGTGAGG CCAAAAGGTC
         E. coli ACAGCAGGCG TATGCCCATA AACCGCTGGT GCGGCTGTAT GACAAAGG..
My. tuberculosis cgaaaaggcc taccatgcag agcctttcat ttatctgatg ccggaggggc
       S. mutans GCAGCATTTC CAGGTGCTGT TCTTGAAGAT GATATTAAAC ATCAAATTTA

                901                                                   950
     B. subtilis AGTACCCGCA AACGAAAGAA GTGTACGGCA GCAATTTCTG TGATATCGCC
         E. coli CGTTCCGGCG CTGAAAAATG TCGTTGGGCT GCCATTT.TG CGATATCGGG
My. tuberculosis AGCTGCCGCG CACCGGCGCG GTGATCGGCA GCAACGCAGC GCACATCGCC
       S. mutans CCCACAAGCA GCGAATGCTG TTGGCAGTCG TACTTTTGTC GGCCGTATTC

                951                                                  1000
     B. subtilis GTGACCCTCG ATGAGAGAAC GAACAGAGTC ACGATCGTCT CGGTAATCGA
         E. coli TTTGCCGTTC AGG......G CGAGCATCTG ATTATTGTGG CGACCGAAGA
My. tuberculosis GTCGCGGTGG ACGAGGACGC GCAGACGTTC GTGGCGATCG CCGCGATCGA
       S. mutans GTAAGGATTT A.GATATTGA AAATGGTATT CATATGTGGG TCGTTTCAGA

                1001                          <<<ARGC2<<<<           1050
     B. subtilis TAATTTAATG AAGGGTGCCG CCGGTCAGGC AGTGCAAAAC TTTAATTTGA
         E. coli CAACTTACTG AAAGGCGCGG CGGCACAAGC GGTACAGTGC GCCAATATTC
My. tuberculosis CAACCTGGTC AAGGGCACCG CCGGCGCCGC GGTGCAATCG ATGAACCTGG
       S. mutans CAATCTTCTT AAAGGTGCTG CTTGGAATTC AATCATCACC GCTAACCGTC

                1051                                                 1100
     B. subtilis TGAATGGCTG GAATGAAGAA ACTGGACTCA CCATCACGCC AATTTATCCA
         E. coli GTTTCGGCTA TGCGGAAACG CAGTCTCTTA TTTAA..... ..........
My. tuberculosis CGCTGGGCTG GCCGGAGACC GACGGCCTTT CGGTTGTGGG GGTGGCGCCG
       S. mutans TACATGAACG GGGTCTTGTT CGTTCGACAT CAGAATTGAA GTTTGAACTG
```

Figure 3.45. PCR amplification of the *erm*-distal flanking chromosomal DNA in mutant 9E. Diagram showing the approach taken to sequence the listerial DNA flanking the *erm*-distal end of the transposon insertion. Primers ERM.DIST and ARGC1, were used to amplify a 350 bp fragment at the *erm*-distal end of the transposon, while primers ERM.DIST and ARGC2 were used to amplify a 800 bp fragment.

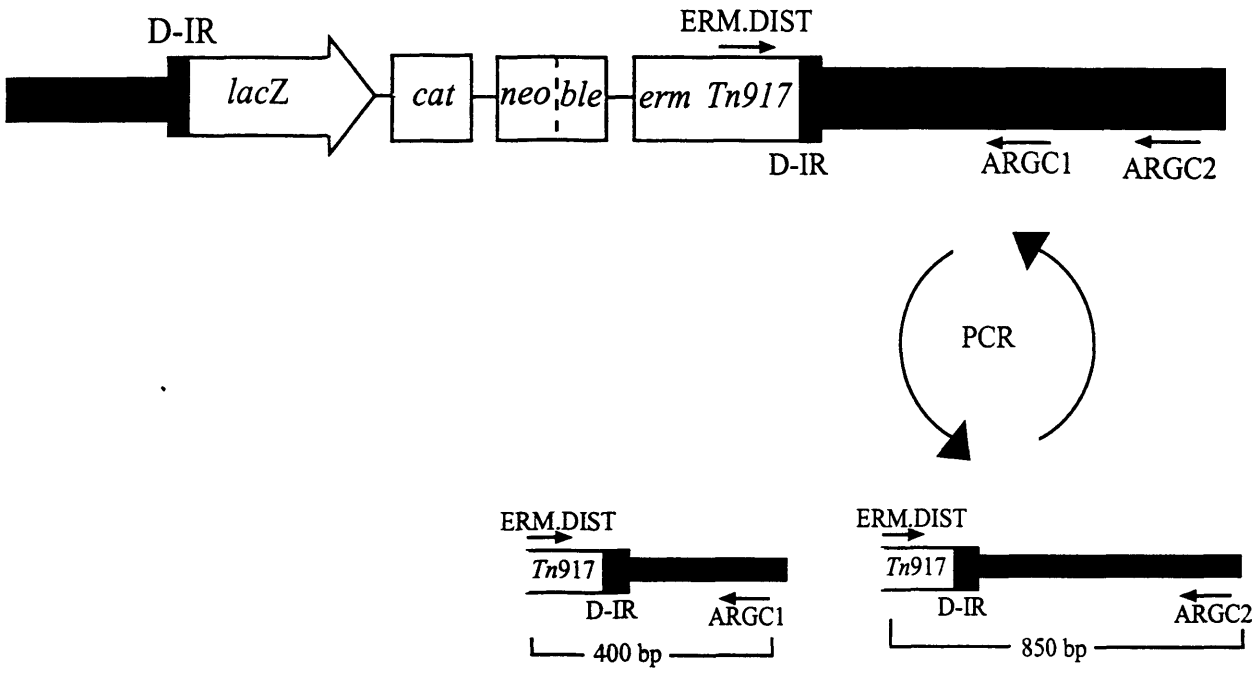
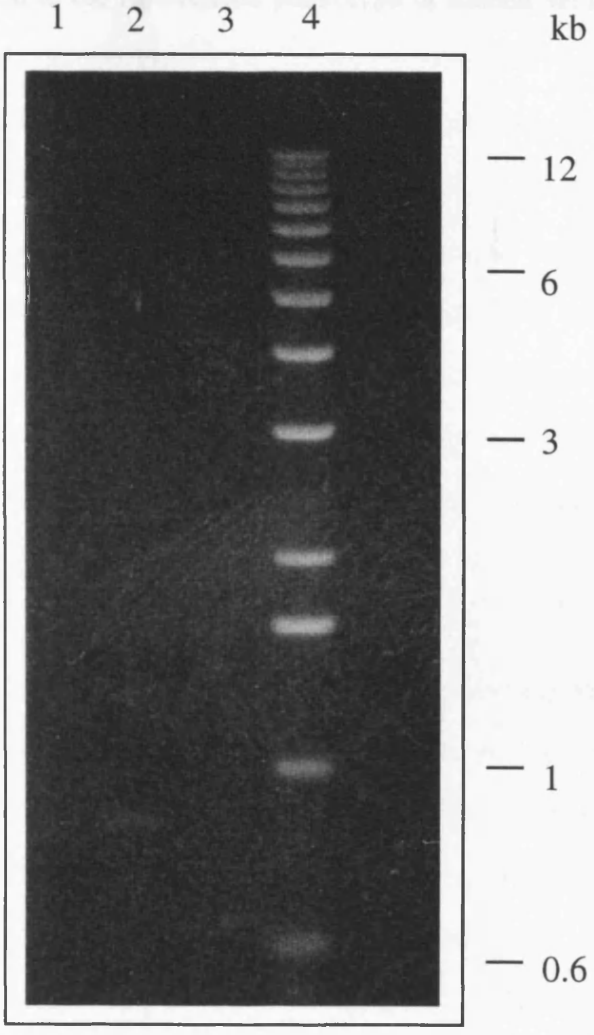

D-IR
lacZ
cat
neo
ble
ERM.DIST
erm Tn917
D-IR
ARGC1
ARGC2
PCR
ERM.DIST
Tn917
D-IR
ARGC1
400 bp
ERM.DIST
Tn917
D-IR
ARGC2
850 bp

Figure 3.46. Gel electrophoresis of PCR products representing the chromosomal DNA at the *erm*-distal side of transposition in mutant 9E. *L. monocytogenes* mutant 9E genomic DNA was used as a template for amplification with ERM.DIST and ARGC1 (lane 1) or ERM.DIST and ARGC2 (lane 2) primers. Wild type genomic DNA was used as a template with ERM.DIST and ARGC2 primers (lane 3). Taq DNA polymerase was used according to manufacturers instructions (GIBCO-BRL). Lane 4 represents 1 kb ladder. The samples were analysed on a 0.7% (w/v) agarose gel.


1
2
3
4
kb
12
6
3
1
0.6

Repeated attempts were made to amplify this 850 bp PCR products to enable subcloning into pCRSriptSK for sequencing. This proved unsuccessful. Due to time limitation, this was not investigated further. Thus, in contrast to the results for mutant 7D, were sequence data was determined for both the *erm*-proximal and the *erm*-distal chromosomal DNA, only sequence data for he *erm*-proximal chromosomal DNA was determined for mutant 9E. However, for the purpose of this investigation, the sequence for the *erm*-proximal chromosomal DNA strongly indicated that in mutant 9E, a putative *argC* gene had been disrupted by transposon insertion. The possible correlation of this mutation to the iron-related phenotype of mutant 9E is discussed in section 4.4.

3.8 Further analysis of the *flaA* listerial mutant (7D) and the putative *argC* listerial mutant (9E)

3.8.1 Determination of a relationship of the *flaA* mutation to the iron-related phenotype of mutant 7D

Having identified the gene which was disrupted by insertion of *Tn*917-pLTV3 in mutant 7D, experiments were performed to investigate this mutant further.

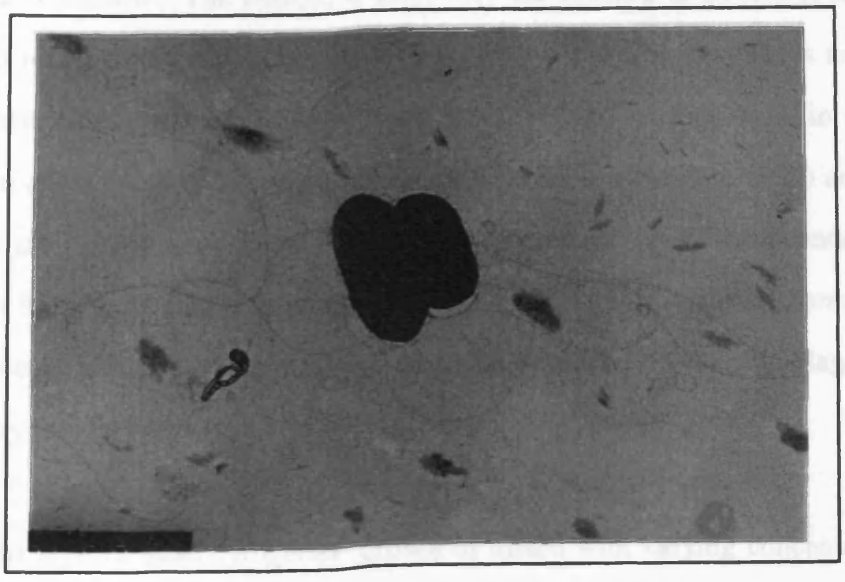
Microscopic examination was performed on mutant 7D, the *flaA* mutant, to assess whether the *flaA* mutation affected the motility of the cell and also to examine whether individual cells possessed a structural flagellin. Phase microscopic examination was performed previously, (Section 3.3, Phenotypic analysis of mutants 7D and 9E) and the results suggested that mutant 7D had markedly reduced motility compared with the wild type strain (*L. monocytogenes*, 10403S) and mutant 9E. This visibly reduced level of motility was noted in section 3.3 and may now be explained by the fact that the gene disrupted in this mutant was in *flaA*, encoding the flagellin structual protein.

Electron microscopic examinations of cultures of mutant 7D and the wild type strain were performed to assess whether flagella were present on the cell. Optimal conditions were used for growing the cells being used for electron microscopic examination of flagella (Kathariou *et al.*, 1995). Cells were grown until logarithmic phase at 22°C in TSB medium and a sample of these cells were used for examination. The electron microscopy was kindly performed by Robert Gilbert (Department of Microbiology, University of Leicester)

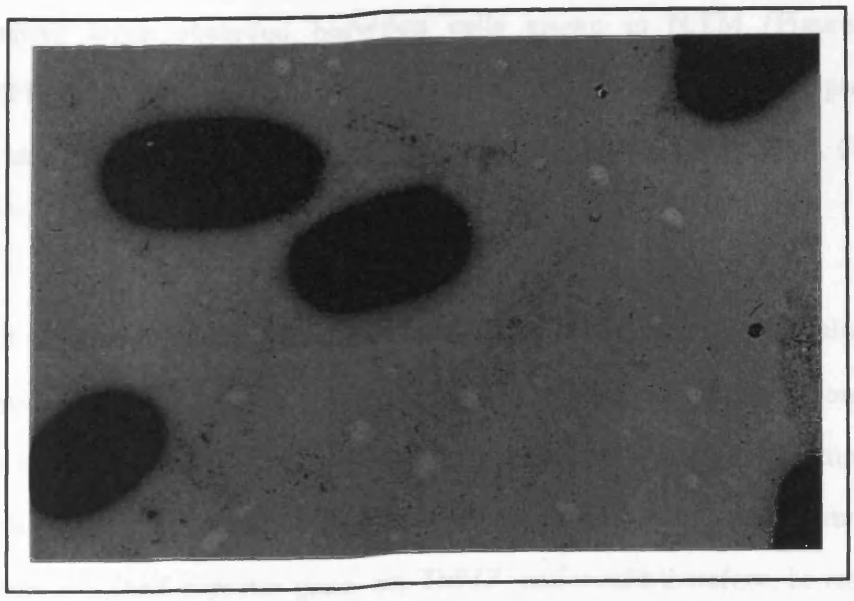
The results are shown in Figure 3.47. Flagella were visible on cells of the wild type strain (Figure 3.47A), while cells from a culture of mutant 7D very clearly, lacked

Figure 3.47. Comparison of electron micrographs of flagella production by *L. monocytogenes* mutant 7D and the wild type (10403S) grown at 22°C in TSB. Electron mircroscopy was performed by R. Gilbert as described briefly in Materials & Methods. Bacterial cells were placed on a carbon coated grid, stained using uranyl acetate and visualized using a Jeol 100-CX transmission electron microscope at an accelerating speed of 100kV. Figure A, magnification factor 10K; Figure B, magnification factor, 14K.

A



B



flagella (Figure 3.47B). This suggested that the *flaA* mutation resulted in the complete lack of flagella on the bacterial surface of cells of mutant 7D.

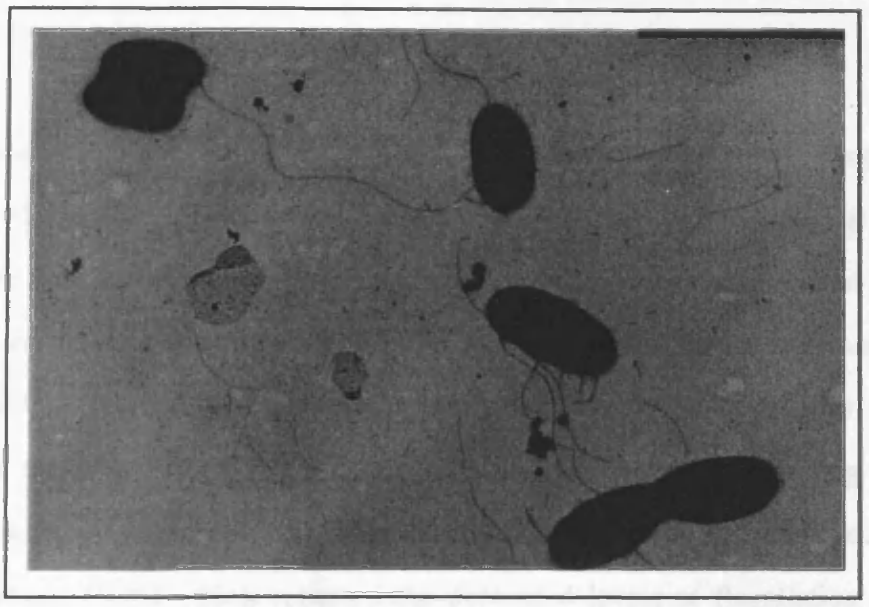
The relationship of this *flaA* mutation to the iron-related phenotype observed in section 3.2 is unclear. The reason, if any, why the absence of structural flagella in mutant 7D resulted in a reduced ability to grow in a low-iron medium is not known although this is discussed further in section 4.3. The fact that flagellin encoding genes from other bacteria including, *B. subtilis* (Chen & Helmann, 1994) and *E. coli* (Guzzo *et al.*, 1991) have been shown to be regulated by environmental iron in addition to the identification in section 3.6 of a Fur-like box upstream from *fla*A led to experiments which investigated whether the presence of *listerial* flagellin was regulated by iron.

Firstly, wild type *L. monocytogenes* grown in media with varying concentrations of iron (N.TM, TM.Fe^{2+} or TM.Fe^{3+}, as described previously in section 3.2) were examined using electron microscopy to assess whether the presence of flagellin was associated with a specific iron concentration. The results are shown in Figure 3.48. No differences were observed between cells grown in N.TM (Figure 3.48A), TM.Fe^{2+} (Figure 3.48B), or TM.Fe^{3+} (Figure 3.48C) suggesting that the presence of flagellin was not affected by growing the bacteria in a low-iron medium, (TM.Fe^{2+} or TM.Fe^{3+}).

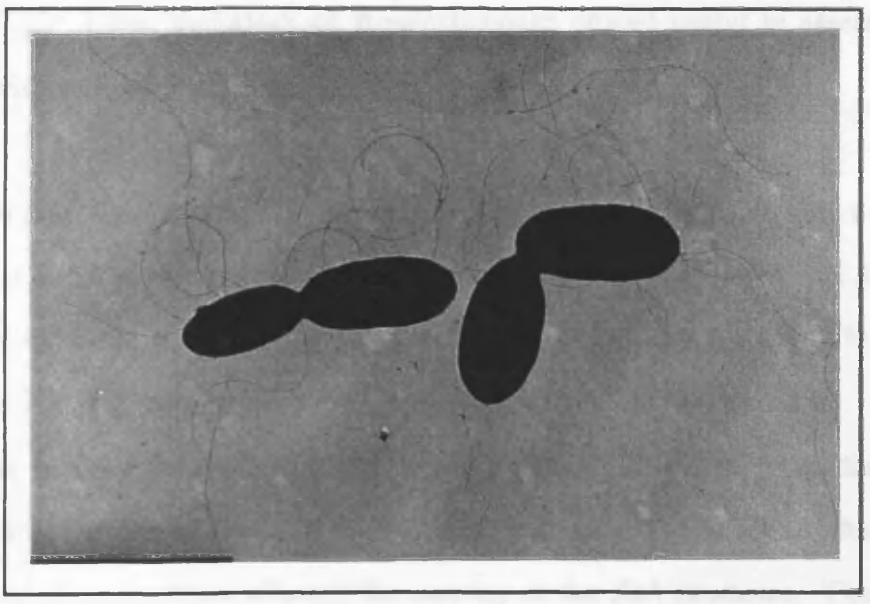
The second approach taken was the examination of the levels of β-galactosidase activity produced by cultures of mutant 7D grown under conditions which represented different levels of environmental iron. From the previous section (3.6), it was determined that the promoter region from the listerial *flaA* gene lay immediately upstream form the *lacZ* reporter gene on *Tn917* and would therefore, be responsible for the expression of *lacZ* in mutant 7D. Thus by examining the level of β-galactosidase produced by mutant 7D grown under various environmental condition,

Figure 3.48. Comparison of electron micrographs of flagella production by *L.monocytogenes* 10403S grown under different iron concentrations. Electron mircroscopy was performed by R. Gilbert as described briefly in Materials & Methods/ Bacterial cells grown at 22°C in N.TM (A), TM.Fe^{2+} (B) and TM.Fe^{3+} (C) were placed on a piolioform-carbon coated grid, stained using uranyl acetate and visualized using a Jeol 100-CX transmission elctron microscope at an accelerating speed of 100kV. Figure A, magnification factor, 10K; Figure B, magnification factor, 10K and Figure C, magnification factor, 33K.

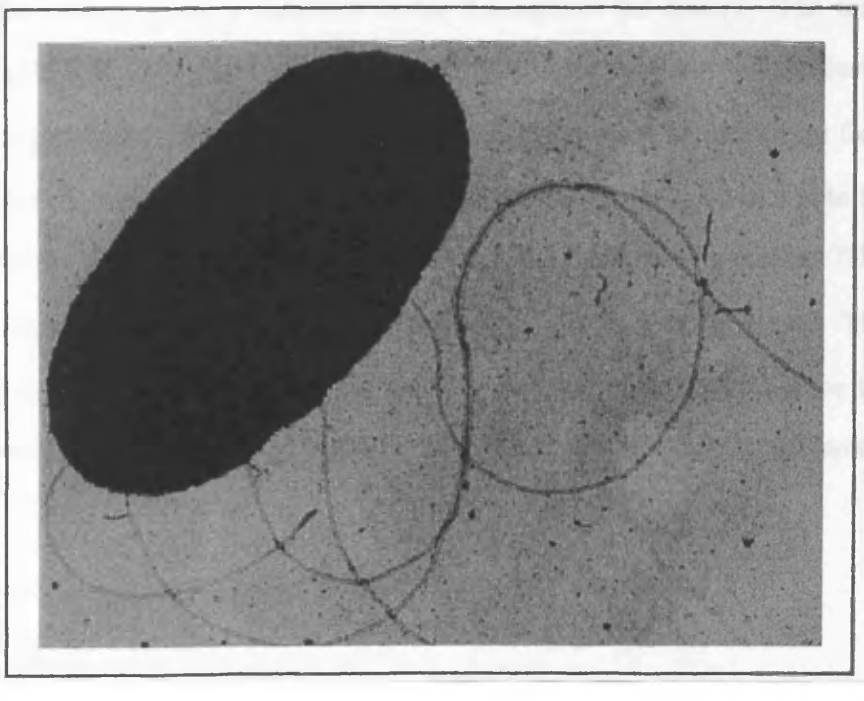
A



B



C



it would be possible to examine the effect of the environmental factors on flagellin expression.

Initially, attempts were made to confirm the previously reported temperature regulation of flagellin expression (Kathariou *et al.*, 1995) by assessing the β-galactosidase activity expressed from cultures of mutant 7D grown in TSB at 22°C or 37°C. The results are shown in Table 3.7 and indicate that cultures of mutant 7D grown at 22°C expressed β-galactosidase levels which were approximately 3 times higher than those produced from cultures grown at 37°C. This finding was in agreement with previous findings (Galsworthy *et al.*, 1990; Dons *et al.*, 1992; Kathariou *et al.*, 1995) which reported the decreased levels of flagellation of cells grown at 37°C. Thus, the assay of β-galactosidase proved useful in assessing the effect of environmental factors on flagellin expression.

Although mutant 7D could not be grown in medium where the iron levels were less than or equal to 20 to 40 μM (Figure 3.8, section 3.2), an experiment was designed where concentrations of 20 μM to 100 μM $FeSO_4$ or $FeCl_3$ were added to TM-Fe and mutant 7D was grown in each of these different media at 22°C. The β-galactosidase activity produced by cultures of mutant 7D grown in medium with different concentrations of ferric or ferrous iron was assayed to examine whether the different iron concentrations affected the activity of the *flaA* promoter. The optical density of the cultures was a constant factor throughout the assays. The results are shown in Table 3.8. No obvious correlation was observed between the level of β-galactosidase produced from mutant 7D grown and the level of ferrous or ferric iron added to TM-Fe. However, it was evident by comparing the data in Table 3.7 with the data in Table 3.8, that the level of β-galactosidase produced by mutant 7D in TSB was approximately 10-fold higher than that produced in TM medium. Thus, the varying iron concentrations in TM -Fe medium did not appear to influence the level of β-galactosidase produced by mutant 7D although an obvious difference was

Table 3.7. β-galactosidase activity expressed by mutant 7D grown in TSB at 22°C or 37°C

TEMPERATURE (°C)	β-GALACTOSIDASE ACTIVITY MILLER UNITS (MU)1
37	997
22	2910

[1]Calculated using the equation of Miller (1972)

Table 3.8. β-galactosidase activity expressed by mutant 7D grown at 22°C in TM-Fe medium with different concentrations of ferrous sulphate or ferric chloride

	β-GALACTOSIDASE ACTIVITY (MU)[1]	
[IRON] μm	FERROUS SULPHATE	FERRIC CHLORIDE
20	174	165
40	205	171
60	47	135
80	179	198
100	303	238

[1]Calculated using the equation of Miller (1972)

observed between cells grown in TM-Fe with additional ferrous or ferric iron and cells grown in TSB. The reason for this latter difference was not known and was not investigated further in this study.

3.8.2. *In vivo* analysis of mutants 7D and mutant 9E

To assess the importance of the *fla*A and *arg*C gene products for *in vivo* survival of *L. monocytogenes*, bacterial counts were taken at day 2, 4 and 6 post-infection, from the spleens of MF1 mice inoculated intravenously with 5 x 10^4 viable organisms of mutant 7D, mutant 9E and the parental strain, *L. monocytogenes*, 10403S. This methodology for assessing the ability of *L. monocytogenes* to survive *in vivo* was described previously (Hof & Hefner, 1988; Stephens *et al.*, 1991).

The results obtained for mutant 7D, mutant 9E and the wild type are presented in Table 3.9. The stastical significance of the differences in counts between the wild type and both mutants at days 2, 4 and 6 was examined using both the T-test and also by Analysis of Variance. In all cases, P>10 which suggested that the differences were not significant. This was suprising, especially for the counts observed at day 4 for both mutants compared with the wild type, where the wild type gave a mean count of 1.7 x 10^4 cfu per spleen, mutant 7D gave a count of 7.2 x 10^3 cfu per spleen and mutant 9E gave a count of 3.4 x 10^2 cfu per spleen. The insignificance of the difference between these counts may be explained by the degree of error calculated for each count (Table 3.9). To clarify this, the experiment would have to be repeated with a larger number of mice, however, from the data presented here it appeared that the *fla*A mutation in mutant 7D and the *arg*C mutation in mutant 9E was not affecting the ability of the mutant to survive *in vivo*. The counts obtained for both mutants on day 2, 4 or 6 were not significantly different from the wild type.

Table 3.9. Bacterial counts per spleen of *L. monocytogenes* wild type, mutant 7D and mutant 9E after intravenous infection

	Counts per spleen[1]		
Days post-infection	Strain		
	Wild type	Mutant 7D	Mutant 9E
2	4.23 (±0.15)	4.23 (±0.16)	3.87 (±0.37)
4	4.58 (±0.65)	3.86 (±1.14)	2.53 (±0.69)
6	1.42 (±0.83)	0[2]	0.8[3]

[1]Expressed as mean log 10/cfu per spleen (±SEM); five animals per group

[2]all mice died; [3]4 mice died

DISCUSSION

4.1 The listerial ferric reductase

The objectives of this investigation were to clone the gene(s) involved in the ferric reductase activity expressed by *L. monocytogenes* and use the gene(s) as the starting point for studying the role of ferric reductase in listerial iron acquisition.

The listerial ferric reductase was first described in 1985 (Cowart & Foster, 1985) as a low molecular weight protein capable of reducing ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}). Although Cowart & Foster (1985) proposed that the ferrous iron was acquired into the cell via a Fe^{2+} binding site on the cell surface, no evidence was provided to support this hypothesis. More recent investigations led to the identification of cofactors which proved necessary for listerial ferric reductase activity (Adams *et al.*, 1990). A colourimetric assay which could be used to detect the presence of this ferric reductase activity was also reported (Deneer & Boychuck, 1993; Deneer *et al.*, 1995).

The colourimetric ferric reductase assay was first described by Dailey & Lascelles (1977) where, a ferric reductase of *Spirillum iternosonii* was identified. The assay was based on the fact that the Fe^{2+} chelator, ferrozine (Stookey, 1970), formed a deep red colour when complexed with Fe^{2+}, the reduced product of the ferric reductase protein. Using this assay, ferric reductases have been identified in other bacterial species including, *Bacillus subtilis* (Gaines *et al.*, 1981), *Streptococcus mutans* (Evans *et al.*, 1986), *Legionella pneumophila* (Johnson *et al.*, 1991) and also in the yeast, *Sacchromyces cerevisiae* (Dancis *et al.*, 1990).

With the exception of the *S. cerevisiae* ferric reductase (Georgatsou & Alexandraki, 1994), no attempts have been made to clone the gene(s) encoding the ferric reductases of the other bacterial species. In *L. monocytogenes* and the previously mentioned bacteria, the investigations have been directed towards determining: (1)

the substrate specificity of the ferric reductases (Moody & Dailey, 1985), (2) the cofactors which are necessary for ferric reductase activity (Dailey & Lascelles, 1977; Johnson *et al.*, 1991), and (3) the effect of environmental factors on the ferric reductase activity (Poch & Johnston, 1993).

Although the investigations to date on the listerial ferric reductase have provided an understanding of what factors are necessary for its activity Deneer & Boychuck (1993), what environmental factors influence its expression by *L. monocytogenes* (Deneer *et al.* 1995), and what substrates it can utilise (Cowart & Foster, 1885; Adams *et al.*, 1990; Hartford *et al.*, 1994), they do not provide evidence of how this protein is involved in the overall iron acquisition by *L. monocytogenes*. Thus, in this present investigation, a molecular biological approach was taken. Attempts were made to isolate from a listerial genomic library in *E. coli*, a clone with a reductase-positive phenotype as determined by the colourimetric assay described by Deneer & Boychuck (1993). Also, this assay was used to screen a library of *L. monocytogenes* transposon mutants for a clone with a ferric reductase-negative phenotype.

The listerial genomic library was constructed using chromosomal DNA from *L. monocytogenes*, 10403S and the vector pTTQ18 (Stark, 1987). The decision to use this vector was based on its availability but also on the characteristic features of this plasmid. These included, a *tac* promoter, a promoterless copy of the *lacZ* gene, an ampicillin resistance marker and a multiple cloning site. The *tac* promoter enabled the expression of the hybrid proteins to be highly regulated (Amann *et al.*, 1983) while the promoterless copy of *lacZ* enabled blue/white selection of recombinants in the presence of the β-galactosidase substrate, X-gal. The library was constructed successfully in *E. coli* Sure and evidence from the analysis of plasmids isolated from 12 random clones suggested that 80% of clones from the library were recombinants. This library was used to screen for a ferric reductase-positive clone.

The *L. monocytogenes* transposon library was constructed using the *Tn*917 derivative *Tn*917-pLTV3 (Camilli *et al.*, 1990). A more detailed description of this derivative is given in section 4.2 below, however, for the purpose of this discussion, it has been shown that *Tn*917-pLTV3 inserts randomly into the chromosome of *L. monocytogenes* and at a high frequency (A. L. Passos, Personal communication). These features made the library suitable for screening for a ferric reductase-negative clone.

In excess of 10, 000 transformants from the listerial genomic library were screened and no ferric reductase-positive clone was identified. This number of transformants was sufficient, as estimated from the Clark & Carbon (1977), to identify a ferric reductase clone from the genomic library. The reasons why no clones were identified are unclear, although it could have been due to a number of factors. The ferric reductase protein may have been toxic to *E. coli* or it could have been that *E. coli* was unable to secrete the protein. The possibility of the ferric reductase being toxic to *E. coli* seems unlikely as it is under the control of the *tac* promoter and therefore its expression is very tightly controlled. However, the possibility that *E. coli* was unable to secrete the ferric reductase seems a more likely explanation, due to the differences between the cell wall of *E. coli* and *L. monocytogenes*. *Escherichia coli*, being Gram-negative, has a 2-membrane cell wall, while *L. monocytogenes*, being Gram-positive, has only one membrane. Thus, it is possible that the listerial ferric reductase was being contained within the cytoplasm or within the periplasmic space of *E. coli*. Given this possibility, future attempts to identify the ferric reductase encoding gene could involve screening the library after a step which would involve lysing *E. coli* to release the ferric reductase if it were present.

Another possible reason why no ferric reductase-positive clones were identified during the screen could have been that the protein was not folded properly. Although no information is available on the three-dimensional folded structure of the listerial

ferric reductase, it is known that the protein requires cofactors, Mg^{2+}, FMN and NADH for activity (Adams, *et al.*, 1990) and presumably would possess binding motifs for these cofactors. Thus, if the ferric reductase protein was not being folded correctly in *E. coli*, it may be unable to bind the cofactors and consequently be unable to reduce ferric iron to ferrous iron.

The approach whereby a library of transposon mutants was screened for a ferric reductase-negative clone had the advantage over the genomic library approach in that it was not dependent on the ferric reductase being secreted by *E. coli* or on the colourimetric assay being sensitive enough to detect recombinant ferric reductase produced by *E. coli*. Instead, the assay was being used to detect a listerial mutant clone which no longer produced a red colony but instead produced a white ferric reductase-negative colony.

The library of transposon mutants was screened extensively and from this initial screen, 11 mutants were identified which produced white colonies indicating a loss of ferric reductase activity. Unfortunately, when these 11 mutants were re-screened they all proved to be ferric reductase-positive, producing red colonies. The reasons for these observations are unknown and were not investigated further as the emphasis of the study switched towards another aspect (Section 4.2, see below). It is possible to speculate that the mutants reverted to having the wild-type phenotype. However the fact that the estimated frequency of reversion of *Tn*917-pLTV3 is approximately 10^{-6} (Camilli *et al.*, 1990) and the fact that the mutants retained their resistance to erythromycin and lincomycin, makes this unlikely. Southern blot data from the 11 mutants before and after the initial screen would be required to prove or disprove this speculation. Alternatively, it may be that the listerial mutants have a second mechanism which becomes induced when other listerial iron uptake genes are disrupted.

Although this investigation has proved unsuccessful in attaining its objectives, it has been the first report of a genetic approach to studying the listerial ferric reductase. It highlighted some of the problems which may be encountered when taking such an approach. It also implied that if a genetic approach were to be taken in the future to study the listerial ferric reductase, it would be more advantageous to take the route of the transposon library or an alternative route altogether, such as constructing a listerial genomic library in a Gram positive bacterium (Youngman, 1987). This would possibly overcome any of the problems of secretion of the ferric reductase. Also, as sequence data becomes available for the ferric reductase proteins of other bacterial species (Gaines *et al.*, 1981; Evans *et al.*, 1986; Johnson *et al.*, 1991), the possibility of using a degenerative PCR approach (Dybvig *et al.*, 1992) would arise.

4.2 Screening for "iron-related" listerial mutants

The objective of this section of the investigation was to identify iron-related *L. monocytogenes* transposon mutants. The definition of iron-related mutants were ones with deficiencies in ability to acquire or utilise iron in low-iron environments compared with the wild-type strain (*L. monocytogenes*, 10403S). Firstly the transposon library of choice had to be constructed and secondly, the medium to be used for screening the library had to be determined.

During the past decade, there has been a surge in the use of transposon mutagenesis for studying the virulence determinants of *L. monocytogenes*. The first report of Gaillard *et al.* (1986) demonstrated the use of the conjugative transposon *Tn*1545 as tool for studying the role of the listerial haemolysin in bacterial virulence. More recent investigations have utilised this same transposon as a means of studying the listerial phosphatidyl choline-specific phospholipase C and the zinc metalloprotease (Mengaud *et al.*, 1991; Raveneau *et al.*, 1992).

Another conjugative transposon, *Tn*916, has also been used successfully to mutagenize *L. monocytogenes* (Kathariou *et al.*, 1987). This study together with more recent studies (*Barry et al.*, 1992; Kuhn *et al.*, 1990; Sun *et al.*, 1990) have demonstrated the reduced ability of *hly*::*Tn*916 mutants to grow intracellularly and to spread from cell-to-cell.

A non-conjugative transposon, *Tn*917, was used by Cossart *et al.* (1989) as a means of studying the listeriolysin. The advantages they found by using this transposon as opposed to the conjugative transposons *Tn*1545 and *Tn*916 were that firstly, it was much smaller in size than the conjugative transposons and consequently, DNA manipulations were more straightforward. Secondly, isolation of transposon mutants was simplified as *Tn*917 was carried by the highly temperature sensitive plasmid

pTV1. Finally, the randomness and frequency of *Tn*917 insertion was greater than those for the conjugative transposons (Cossart *et al.*, 1989).

In this study, the library of *L. monocytogenes* transposon mutants was constructed by A.L. Passos (Dept of Microbiology & Immunology, University of Leicester) using a derivative of *Tn*917, namely, *Tn*917 - pLTV3 (Camilli *et al.*, 1990). This derivative has been used recently in *L. monocytogenes* to study the intracellular behaviour of *hly*::*Tn*917-pLTV3 mutants (Sun *et al.*, 1990).

Camilli *et al.* (1990) designed this *Tn*917 derivative such that it included a number of features which made it more favourable to use over the other transposons which have been used in *L. monocytogenes*, *Tn*1545 and *Tn*916. These features included: (1) a highly temperature sensitive origin of replication on the carrying plasmid pLTV3 (2) a promoterless copy of the *E. coli lacZ* gene (3) the antibiotic resistance gene, which encodes chloramphenicol resistance (*cat*) in *E. coli* and the genes *neo* and *ble* which encodes neomycin and bleomycin resistance in *L. monocytogenes* (4) the ColE1 and M13 origins of replication and (5) a multiple cloning site. All these features enabled the easy selection of chromosome insertions, the generation of transcriptional *lacZ* fusions, and the easy recovery in *E coli,* of chromosomal DNA adjacent to the *erm*-proximal side of the transposon insertion. A comprehensive review of the conversion of *Tn*917 to its *Tn*917-pLTV3 is given by Youngman (1990).

The work by Camilli *et al.* (1990) demonstrated that *Tn*917-pLTV3 transposed into the listerial chromosome with a frequency of 8.2×10^{4} which was similar to the transposition frequency determined for the library used in this work (A.L. Passos, Personal communication). The transposition frequencies determined for *Tn*1545 (Gaillard *et al.*, 1986) and *Tn*916 (Kathariou *et al.*, 1987) were approximately 10000 and 100 times less than that for *Tn*917-pLTV3, respectively.

The randomness of insertion of *Tn*917-pLTV3 into the listerial chromosome was also examined by Camilli *et al.* (1990). They found that although hotspots for insertions did exist, namely in the *hly* gene, the insertions outwith this region were sufficiently abundant and random to allow very effective mutagenesis using this derivative. In this investigation, the randomness of insertion was examined by A.L Passos. Using a probe derived from pLTV3 she demonstrated that out of 12 randomly selected mutants only three showed transposition in the same sites as evident from the same sized bands on a Southern blot. The other nine mutants gave rise to different sized bands, indicating the transposon had inserted at a different location on the chromosome of these mutants (unpublished observations). Thus, the library of *Tn*917-pLTV3 *L. monocytogenes* which was to be screened in this study, consisted of enough random mutants which could be used for for screening for a particular phenotype; in this case the iron-related phenotype.

The second stage in this section of the work was to determine which conditions were suitable for screening the transposon library for mutants with a deficiency in their ability to acquire or utilise iron compared with the wild-type strain (*L. monocytogenes* 10403S). The approach which was taken was to compare the ability of the mutants with the ability of the wild-type to grow in iron-poor or iron-rich medium. Previously, attempts to identify iron-related mutants have been made in *L. monocytogenes* (Roquette *et al.*, 1996) and also in other bacterial species, including *Legionella pneumophila* (Pope *et al*, 1996), *Vibrio anguillarum* (Chen & Helmann. 1994), *Vibrio cholera* (Sigel *et al.*, 1985; Amaro *et al.*, 1995), *Pseudomonas aeruginosa* (Sokol 1987) and *Streptococcus pneumoniae* (Tai *et al.*, 1993). These investigations reported the use of the iron chelator, ethylenediamine di(*o*-hydroxy phenylacetic acid) (EDDHA; Miles & Khimji, 1975) to obtain an iron-poor medium. In all the investigations described above, the minimum inhibitory concentration (MIC) of EDDHA was determined. The MIC defined as the minimum concentration

of chelator which inhibited bacterial growth in a manner which could be reversed by the addition of iron (Aznar *et al.*, 1989; Pope *et al.*, 1986).

The use of EDDHA for removing iron from TSB had been reported previously (Deneer & Boychuck 1993; Deneer *et al.*, 1995), and led to experiments in this present study to determine the MIC of EDDHA required to inhibit listerial growth in TSB. Although the investigation of Deneer & Boychuk (1993) reported that 1.3 mM EDDHA was sufficient to remove the free iron from TSB, the result of this present study suggested that the minimum concentration which inhibited listerial growth in an iron-reversible manner was 8 times higher than 1.3 mM. Concentrations below 10.4 mM were not able to inhibit listerial growth, while concentrations above 10.4 mM were shown to inhibit listerial growth, but in a manner which could not be reversed by the addition of iron. The level of growth inhibition observed by the addition of 10.4 mM to TSB was significant but growth was not completely abolished. The overnight cultures gave OD_{600} of 0.4 to 0.5 in the absence of iron suggesting that not all iron was removed and was thus supporting this reduced growth. As discussed, this residual iron could not be removed by addition of more EDDHA since a concentration > 10.4 mM presumabley removed other essential ions.

As the screening procedure for the iron related mutants was to involve the observation, by eye, of growth or no growth in iron-poor and iron-rich medium, this residual growth in TSB would make this an impossible task. Therefore, an alternative strategy was taken and involved the investigation of another iron chelator, namely, tropolone. Its ability to convert TSB to an iron-poor medium was assessed. This chelator was examined recently (Vidon & Spreng 1992) and the MIC was reported as being 0.048 mM.

The MIC of tropolone in this present study was similar to that of Vidon & Spreng (1992). However, the fact that different batches of TSB required different MIC of

chelator (0.024 mM for Batch A and 0.048 for Batch B in this present study), again made the use of this type of medium as an iron-poor medium impractical. The necessity for determining a new MIC of chelator for each new batch of medium would prove too time consuming.

Thus, the final approach and fortunately the successful approach taken, to determine an iron-poor medium, for screening for iron-related mutants, involved the use of a chemically defined medium of Trivett & Meyer (1971). This medium was recently shown to be the most favourable defined medium for the growth of *L. monocytogenes* (Jones *et al.*, 1995). The initial reason for opting for the use of iron chelators with a complex medium such as TSB rather that a defined medium was that it would minimise the likelihood of identifying auxotrophic mutants as iron-related mutants. The use of a chemically defined medium could result in the identification of such false positives. However, due to unanticipated problems encountered in the iron chelator studies, the use of Trivett & Meyer's chemically defined medium (TM) appeared to be the way forward.

The results reported here, indicated that omission of iron from TM resulted in complete inhibition of growth of the wild-type strain (*L. monocytogenes*, 10403S). A similar finding was reported by Vidon & Spreng (1992). where the removal of $FeCl_3$ from Welshimer's defined medium (Welshimer, 1963), also inhibited listerial growth. They also showed that the addition of 1.0 mM $FeCl_3$ to this iron-free Welshimer's medium was sufficient to support listerial growth.

In this present investigation, a range of $FeSO_4$ and $FeCl_3$ concentrations were added to iron-free TM medium. This enabled the determination of the minimum concentration of $FeSO_4$ and $FeCl_3$ required to support listerial growth in a level similar to that observed in unaltered TM (Jones *et al* , 1995) . The results indicated that as little as 1 μM $FeSO_4$ or $FeCl_3$ was sufficient enough to allow the OD_{600} after

24 hr to reach a similar level to that reached in unaltered TM. This level of iron was 100 times less than the value that Vidon & Spreng (1992) reported as being required for listerial growth in Welshimer's medium. Comparing investigations in other bacterial species (Hantke, 1987; Huyer & Page, 1989; Evans *et al.*, 1986) where different media were used, it is evident that the minimum concentration of iron required for bacterial growth is variable between species, although the concentration required is usually micromolar. The variation could also be due to the type of medium used for growth of the bacterium. Thus, the iron-poor medium used for screening the library of *L. monocytogenes Tn*917-pLTV3 mutants was TM medium lacking the normal levels of $FeCl_3$ but containing 1 μM $FeSO_4$ or 1 μM $FeCl_3$. Growing the bacteria in low-iron medium would allow analysis of iron acquisition mechanisms which are important for listerial growth in low-iron environments such as in mammalian hosts. If high iron concentrations were used, it may not have been possible to investigate such mechanisms as they may not have been required for growth of the bacterium in high-iron environments. TM.Fe^{2+}/Fe^{3+} media were chosen over TSB-EDDHA or TSB-tropolone due to the fact that the EDDHA concentration which completely inhibited listerial growth was also likely to be removing other essential ions and the amount of tropolone which was required to be added was found to be highly variable.

The initial screen for iron related mutants involved analysis of the growth of approximately 5,500 mutant in TM-Fe, TM.Fe^{2+} (TM-Fe + 1 μM $FeSO_4$). TM.Fe^{3+} (TM-Fe + 1 μM $FeCl_3$) and N.TM (unaltered TM). From this screen, 44 mutants were identified which did not grow in TM.Fe^{2+} and TM.Fe^{3+}. 28 of these 44 mutants were auxotrophs, as they did not grow in N.TM but grew normally in the complex medium TSB, and they were excluded from further study.

Upon re-examination, 2 of the 16 mutants possessed the phenotype which was being sought, while the other 14 mutants grew as the wild type. The two mutants of interest

were designated 7D and 9E and both showed visibly reduced growth in TM.Fe^{2+} and TM.Fe^{3+} compared with the wild-type strain which grew as if in N.TM in both these media. In N.TM, mutants 7D and 9E grew in a manner similar to the wild-type. The fact that the only difference between N.TM and TM.Fe^{2+} or TM.Fe^{3+} was the levels of iron, suggested that mutant 7D and mutant 9E were iron-related mutants.

Previously, mutants sensitive to the levels of environmental iron have been identified in *L. monocytogenes* (Roquette *et al.*, 1996), and also in other bacteria including *Legionella pneumophila* (Pope *et al.*, 1996), *Vibrio species* (Chen *et al.*, 1994; Sigel *et al.*, 1985), *Ps. aeruginosa* (Sokol, 1987). The nature of the iron-related defects in the *L. monocytogenes* mutants of Roquette *et al.* (1996) and the *L. pneumophila* mutants of Pope *et al.* (1996) was not determined although the iron-related defects described in the *Vibrio* species and in *Ps. aeruginosa* were shown to be due to alterations in siderophore production and transport.

Thus, the subsequent work in this present investigation was aimed at determining the nature of the iron-related defects which were occurring in the listerial mutants 7D and 9E.

4.3 Analysis of mutant 7D

This section discusses the investigations performed on mutant 7D at the phenotypic and genetic level. A number of phenotypic characteristics of this mutant were examined and compared with the wild-type to determine whether mutant 7D differed from the wild-type in characteristics other that the iron-related phenotype. At the genetic level, chromosomal DNA flanking the *erm*-proximal and *erm*-distal end of the transposon was sequenced and led to the identification of the gene which was disrupted in this mutant.

Initial phenotypic analysis revealed 4 characteristic differences between mutant 7D and the parental strain, *L. monocytogenes*, 10403S. These were, (1) visibly reduced motility, (2) increased levels of haemolytic activity, (3) enhanced β-galactosidase activity and (4) resistance to erythromycin and lincomycin, compared with the wild type.

From the β-galactosidase data for mutant 7D, it was possible to speculate, at this stage, that the *Tn*917 in mutant 7D had inserted downstream from an active listerial promoter. This was confirmed in this study and is discussed below. The resistance of mutant 7D to erythromycin and to lincomycin confirmed the presence of *Tn*917 in the chromosome of the mutant; the parental wild type had no antibiotic resistance characteristics. The reduced motility and the increased haemolytic activity of mutant 7D are discussed in more detail below.

No other phenotypic differences were revealed. The colony morphology, fermentative capacity towards numerous carbohydrates on API-50CH tests, enzymatic activity towards various substrates on API-20E tests, ferric reductase activity or SDS-PAGE protein profiles were examined. No conclusion was drawn from the one-dimensional SDS-PAGE profiles but no gross differences were

observed. More discriminating analysis of protein profiles using techniques such as silver staining or two-dimensional electrophoretic analysis would have to be performed to reach a firm conclusion regarding the absence or presence of particular proteins due to the mutations in mutant 7D or mutant 9E. This was not investigated further in this present study although the possibility of investigating this further exists.

Southern blot analysis of DNA from mutant 7D (and mutant 9E) led to the construction of partial restriction endonuclease maps of chromosomal DNA flanking the *erm*-proximal side of transposon insertion. They also confirmed that transposition had occurred only once and that it had occurred legitimately. The knowledge of the presence of specific restriction endonuclease sites on the flanking chromosomal DNA enabled the most suitable enzyme to be chosen for digesting the genomic DNA for rescuing the flanking DNA directly into *E. coli* (Camilli *et al.*, 1990). An alternative approach, called, "shotgun cloning" of chromosomal DNA flanking transposon insertions (Youngman, 1987), could have been used. This would have involved choosing random enzymes and assuming they would digest the flanking chromosomal DNA at a region near the erm-proximal end of the transposon. However, in this investigation it was decided to construct a partial restriction map of the flanking chromosomal DNA as it would be advantageous to have knowledge of the restriction endonuclease sites in the flanking chromosomal DNA further into the investigation where subcloning of specific DNA fragments and sequencing was performed.

Confirmation of legitimate transposition of *Tn*917-pLTV3 in mutant 7D (and mutant 9E) was made because of the previous observation of illegitimate transposition of *Tn*917-pLTV3 into the listerial chromosomal (A. Passos, personal communication; Alexander 1994). The results of the Southern blots using the probes mentioned above and also using an additional probe based on a region of pLTV3 which included the

tetracycline resistance gene led to the conclusion that correct, legitimate transposition had occurred in this mutant.

Taking advantage of the partial restriction maps which had been constructed for the chromosomal DNA flanking the *erm*-proximal side of insertion in mutant 7D and mutant 9E, DNA flanking this side of the transposon was cloned into *E. coli* MC1061. This approach was described previously by Camilli *et al.* (1990) and Sun *et al.* (1990).

As demonstrated in this present study and also in other investigations (Youngman, 1989; Guitterez *et al.*, 1996), the successful recovery of chromosomal DNA flanking *Tn*917 derivatives such as *Tn*917-pLTV3, which contained the *erm* gene, depended largely on using an *E. coli* strain such as MC1061. Youngman (1989) speculated that the *erm* gene product may be toxic to *E. coli* strains because of its ability to methylate ribosomal RNA. Strains such as MC1060 contain a *rspL* mutation which lead to alteration of the ribosome such that streptomycin resistance is conferred. This alteration was thought to also prevent methylation by the *erm* gene product (Guitterez *et al.*, 1996) and thus, would allow the DNA fragments containing the *erm* gene to be rescued.

The plasmid recovered from mutant 7D in *E. coli* contained chromosomal DNA from the *erm*-proximal end of the transposon and was designated pJM1. Subcloning of different fragments of flanking chromosomal DNA into pBluescript and sequence analysis, revealed that the region which had been disrupted in mutant 7D was significantly homologous to the previously published listerial *flaA* gene (Dons *et al.*, 1992). The *flaA* gene in *L. monocytogenes* encodes the structural flagellin protein and the insertion of *Tn*917-pLTV3 occurred at nucleotide 295 of this gene. This region of *flaA* lies downstream from the transcription initiation site and the -10 and -35 consensus sequences which show homology to *E. coli* promoter regions. Further

confirmation that the *flaA* was disrupted in this mutant was achieved by obtaining sequence data for the chromosomal DNA flanking the *erm*-distal end of the transposon. As expected, the deduced amino acid sequence from the *erm*-distal chromosomal DNA was homologous to the published amino acid sequence of FlaA (Dons *et al.*, 1992).

Having identified the gene which had been disrupted in mutant 7D, it was important to consider whether the inability of this mutant to grow in low-iron medium, the visibly reduced level of motility and the increased haemolytic activity were a consequence of the *flaA* mutation or whether these phenotypic differences of mutant 7D were the result of a point mutation elsewhere on the chromosome or due to a polar effect of the mutation on a downstream gene.

Unfortunately, time limitation did not allow experiments to be performed to directly clarify which of the above situations was responsible for the phenotypes of mutant 7D. However, had time permitted, attempts to complement the mutation with the wild type *flaA* gene could have been made. Had the wild type gene complemented the mutation in mutant 7D such that it could grow in low-iron medium and had levels of haemolytic activity and motility similar to the wild type, it could have been concluded that the *flaA* mutation was responsible for the inability of mutant 7D to grow in the low-iron medium. However, if no complementation was observed, the conclusion which would have been reached was that the *flaA* mutation was not responsible for the observed phenotypes and they were most likely due to a polar effect or a point mutation elsewhere on the chromosome.

Supportive evidence of the involvement of the *flaA* in causing the phenotypic differences of mutant 7D came from the existence of a separate listerial *flaA* mutant made by insertional mutagenesis of *L. monocytogenes* (provided by Dr C. Rees, University of Nottingham). The finding that this second *flaA* mutant also had an

inability to grow in low-iron medium (data not shown) confirmed that this phenotype was related to the disruption of the *fla*A gene. Interestingly, the *fla*A mutant of Dr C. Rees was also shown to have increased levels of haemolytic activity, a phenotype also observed for mutant 7D.

The abilities of mutant 7D, the *flaA* mutant of Dr C. Rees and the parental wild types of both mutants to survive *in vivo* were examined. Intravenous infection of MFI mice and bacterial counts from the spleens of the mice over the period of 6 days was performed. The results obtained for mutant 7D and its wild type (shown in this study, section 3.8) and for the mutant and wild type of Dr C Rees (results not shown in this study) were similar. The difference between the bacterial counts obtained for both mutants and their respective wild type parent over the period of 6 days was not statistically significant. This suggested that the *flaA* mutation was not affecting the ability of the bacterium to survive *in vivo*. Previous investigations (Galsworthy, 1990; Dons *et al.*, 1992; Kathariou *et al.*, 1995) reported the lack of flagellar expression at 37°C (*in vivo* temperature) and also speculated that flagellin was not a important factor for *in vivo* survival. However, not only was mutant 7D a flagellin-minus mutant, it also was shown to have increased haemolytic activity and also an inability to survive in low-iron environments.

The *in vivo* analysis suggested that the increased haemolytic activity of mutant 7D, did not affect the ability of the organism to survive *in vivo*. The mutant produced bacterial counts over the period of the *in vivo* experiment which were not significantly different from the parental wild type. The factor which was causing this haemolytic activity was not defined in this study. Whether it was due to increased production of listeriolysin (Lui & Bates, 1961) was not investigated, although further investigations such as extracellular protein preparations from mutant 7D and the wild type and immunoblotting with listeriolysin-specific monoclonal antibody would qualitatively clarify if more listeriolysin was being produced by mutant 7D than by

the wild type. Kathariou *et al.*, (1990) reported that levels of listeriolysin activity were not related to virulence. Therefore, if the haemolytic producing factor of mutant 7D did prove to be the listeriolysin, no effect on the bacterial counts of mutant 7D as a result of the increased haemolytic activity would have been be expected.

The data from the *in vivo* experiment, also, suggested that the inability of this mutant to grow in low-iron medium *in vitro*, did not affect the ability of the organism to grow and survive *in vivo*. Previous investigations of other bacterial species (Montgomerie *et al.*, 1984; Cox, 1982) have reported the reduced virulence *in vivo* of strains which have been shown, *in vitro*, to have a deficiency in their ability to acquire iron. Thus, the results observed in this present study for mutant 7D (and mutant 9E) were unexpected. The inability of mutant 7D (and mutant 9E) to grow in low-iron *in vitro* did not affect their ability to grow *in vivo*. An understanding of the mechanism by which this *flaA* mutation was resulting in mutant 7D having an inability to grow in low iron would allow a better understanding of why no affect on *in vivo* behaviour was being observed as a result of this phenotype.

The mechanisms by which the *flaA* mutation was causing the phenotypic differences of mutants 7D were not known. It is interesting to speculate that the absence of structural flagellin from the mutant was causing the bacteria to become leaky and consequently allowing iron and the haemolysis producing factor to be exported from the cell via the flagellin export pathway. The flagellin export pathway is not fully understood and whether the proteins which pass through it are required to possess a specific recognition signal is not known (MacNab, 1996). Therefore, it may be that the flagellin export pathway in mutant 7D was allowing leakage of cytoplasmic components of the cell into the extracellular environment. A qualitative comparison of the levels of other secreted proteins, such as PI-PLC (Goldfine & Knob, 1992), metalloprotease (Domann, *et al.*, 1991) and lecithinase (Geoffroy *et al.*, 1991), produced by mutant 7D and the wild type, using monoclonal antibodies specific for

the individual proteins to immunoblot the protein preparation, would allow clarification of the above theory.

The reason why mutant 7D was unable to grow in low-iron medium but was able to grow in high-iron medium was unclear but, it may have been due to the fact that the amount of iron in high-iron medium was overcoming the presumed deficiency of mutant 7D to maintain cytoplasmic iron at levels which were required for bacterial growth. Perhaps the excess amounts of iron in the high-iron medium would enable iron acquisition to occur at a rate similar to the rate of iron loss from the leaky bacterium, whereas, in low-iron medium, iron loss was more rapid than iron acquisition and consequently resulted in an inability of the bacterium to grow. This could be examined by measuring the rates of acquisition of radiolabelled iron by *L. monocytogenes* grown in the high- and low-iron media. No definite conclusion was reached regarding the mechanism by which the *flaA* mutation was causing the observed phenotypes.

Interestingly, a region was identified upstream from the -10 and -35 consensus sequences of *fla*A which had significant homology to the Fur box consensus sequence of *E. coli* (de Lorenzo *et al.*, 1988; Griggs & Konisky, 1989) and other bacterial species (Tai & Holmes, 1988; Stojiljkovic *et al.*, 1994). Fur boxes are sequences found in the promoter region of various genes which result in iron-regulated expression of the downstream gene (Hennecke, 1990). Binding of the Fur protein to the Fur box under conditions of high iron results in repression of the downstream gene. The finding, in this study, of a Fur-like box upstream from *fla*A suggested that expression maybe influenced by iron. Further evidence which suggested that *flaA* expression may be influenced by iron was the finding of σ^{28} promoter sequences upstream from the *fla*A gene both in this study and in the report of Dons *et al.*, (1992). In *B. subtilis*, the chemotactic/flagellin encoding genes which were preceded by σ^{28} consensus sequences were shown to be repressible byiron (Chen & Helmann,

1994). Iron-regulation of flagellin-associated genes was also observed in *E. coli* (Guzzo *et al.*, 1991) and *Vibrio parahaemolyticus* (McCarter & Silverman, 1990).

The occurrence of β-galactosidase activity in mutant 7D was exploited to investigate the effect of environmental stimuli on the activity of the *fla*A promoter. The effect of temperature on β-galactosidase expression was examined by growing mutant 7D at 22°C or 37°C. This was examined due to the previous reports that flagellin expression was regulated by environmental temperature (Galsworthy *et al.*, 1990; Kathariou *et al.*, 1995)). The results in this study showed a 3-fold increase in β-galactosidase expression from cells grown at 22°C compared with cells grown at 37°C. This observation was consistent with previous observation that flagella production by cells grown at 37°C was less than by those grown at 4°C (Kathariou *et al.*, 1995). Previous electron microscopic examination of *L. monocytogenes*, 10403S cultured at 22°C or 37°C demonstrated the temperature regulation of flagellin expression, were cells grown at 37°C did not appear to have structural flagella while those grown at 22°C did (Galsworthy *et al.*, 1990).

An investigation of the effect of different concentrations of iron on the expression of the *fla*A gene was undertaken both using electron microscopy and also by assaying β-galactosidase. No changes in β-galactosidase levels with changes in environmental iron concentration was seen. Electron microscopic examination of the wild-type strain grown in TM.Fe2+, TM.Fe^{3+}, or N.TM also did not show any variation in flagella production with variation in iron. These observations were unexpected, especially after the finding of a Fur-like box and a σ^{28} consensus region in the promoter region of the *fla*A gene. The presence of both these motifs would suggest that expression of *fla*A was regulated by environmental iron. This does not seem to be the case here.

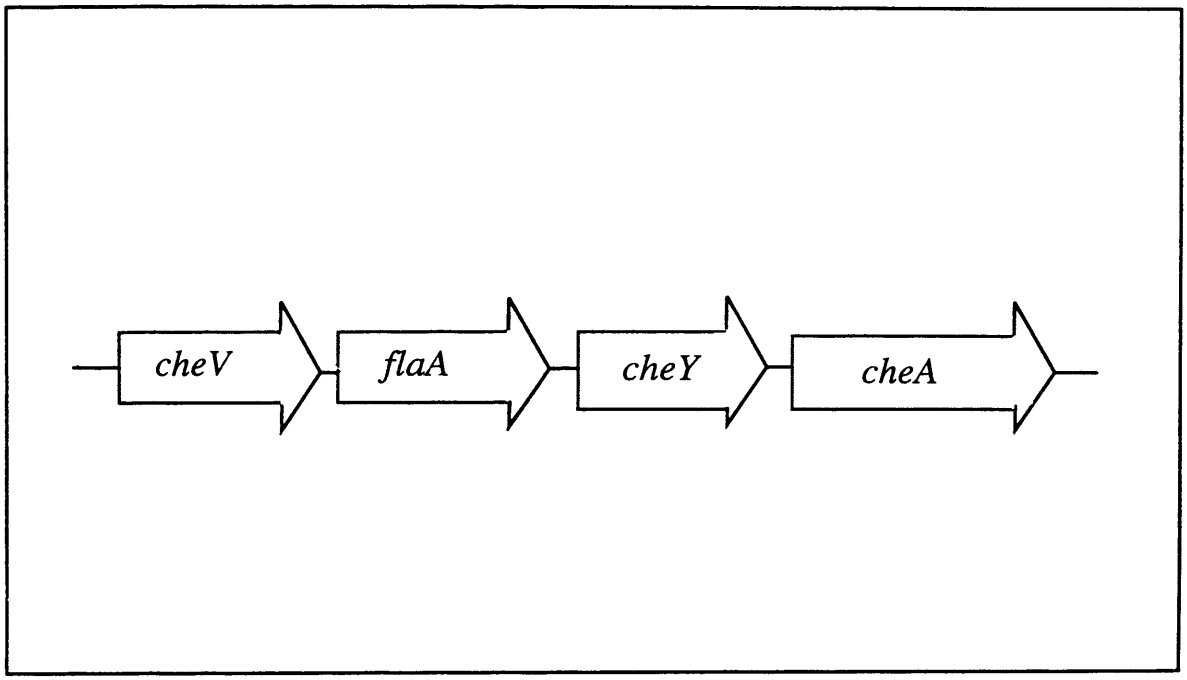
Sequence analysis of the region upstream from the *flaA* promoter region in mutant 7D led to the identification of a novel *listerial* gene. It was designated *cheV* as it was shown to have significant identity at the amino acid level to the *cheV* gene of *B. subtilis* (Fredrick & Helmann, 1994). CheV, in *B. subtilis*, is a protein which is involved in regulation of the complex process of bacterial chemotaxis (MacNab, 1987). Chemotaxis in *B. subtilis* and in other bacterial species results from the modulation of the direction of flagellar rotation in response to environmental stimuli. The chemotactic system is composed of a number of proteins, including the Che proteins (MacNab, 1987). CheY, the response regulator protein, becomes phosphorylated by the auto-phosphorylated kinase protein CheA, in response to various environmental stimuli. Phosphorylated CheY then acts directly on the flagellar motor to influence bacterial movement. Other Che proteins include; CheW (increases the rate of CheA auto-phosphorylation); CheZ (removes phosphate from CheY); CheB (phosphorylated by CheA and acts directly on membrane flagellar proteins) and CheR (methyl transferase which acts on membrane flagellar proteins). A description of the interaction of these different proteins with each other and a more detailed description of their role in chemotaxis is given in the recent review of MacNab (1987).

The putative listerial CheV protein described in this study appears to be similar to the *B. subtilis* CheV protein (Fredrick & Helmann, 1994) in that it has two domains; one with homology to the CheY, response regulator protein and one with homology to the CheW protein which acts on the kinase protein CheA to increase the rate of auto-phosphorylation. Recently, CheY and CheA proteins have been identified in *L. monocytogenes* (Dons *et al.*, 1994) and the encoding genes mapped to the region immediately downstream from the *fla*A gene. Thus, from the data of Dons *et al.*, (1994) and the finding of this study of a CheV encoding gene upstream of *fla*A, it appears that the chemotactic genes of *L. monocytogenes* occur in a cluster which is similar to the situation in *B. subtilis*, *E. coli* and *S. typhimurium* (Fredrick &

Helmann, 1994; Rosario *et al.*, 1994; Guzzo *et al.*, 1991; MacNab, 1987). The arrangement of these *listerial* genes in *L. monocytogenes* is shown in Figure 4.1.

No Fur-like consensus boxes were identified in the promoter region of the *che*Y or *che*A genes described previously (Dons *et al.*, 1992). The *che*V gene described in this study also did not appear to have a Fur-like box upstream. However, consensus sequences of σ^{28} promoters were identified upstream from the *che*Y gene and the *che*A gene (Dons *et al.*, 1994) suggesting that their expression may be influenced by iron. No σ^{28} consensus sequences were identified upstream from the *che*V gene described in this study.

Figure 4.1 Arrangement of *flaA* and chemotactic genes on the chromosome of *L. monocytogenes*. Diagrammatic representation of the arrangement of *flaA*, *cheY*, *cheA*, and *cheV* of *L. monocytogenes* deduced from the work in this study and by Dons *et al.*, (1992). The arrow indicates the direction of transcription.


cheV
flaA
cheY
cheA

4.4 Analysis of mutant 9E

Phenotypic and genotypic characterisation of mutant 9E was undertaken in this study. The colony morphology, fermentative capacity on various carbohydrates on API-50CH tests, the enzymatic activity on a range of substrates on API-20E tests, haemolytic activity on blood agar, ferric reductase activity, and SDS-PAGE protein profiles of mutant 9E were compared with those of the wild-type strain (*L. monocytogenes*, 10403S) and no differences were observed. In contrast to the finding of β-galactosidase activity from mutant 7D, no activity was expressed by mutant 9E. This suggested that transposition may have occurred at a region of the chromosome which was not in the vicinity of an active gene promoter. Thus, the only phenotypic characteristic of mutant 9E which differed from the wild-type strain, besides resistance to erythromycin and lincomycin, was the inability of mutant 9E to grow in low-iron medium.

Initial genotypic investigations of mutant 9E undertook an identical path as discussed for mutant 7D (section 4.3). Southern blot analysis of chromosomal DNA from mutant 9E using probes derived from pLTV3 led to the construction of a restriction endonuclease map of the *erm*-proximal flanking DNA, in addition to the conclusion that legitimate transposition of one copy of *Tn*917 in the chromosome of mutant 9E had occurred. Following cloning, sequencing and amino acid databases comparisons using BLASTX (National Centre for Biotechnology Information, Los Alamos, N. MEX) of the *erm*-proximal flanking DNA, an open reading frame (ORF) immediately flanking the *erm*-proximal side of the transposon was identified and was designated ORFB. ORFB was shown to have significant identities and similarities to the N-terminus of the ArgC protein of *B. subtilis* (Smith *et al.*, 1990), *E. coli* (Parsot *et al.*, 1988), *S. mutans* (Cardineau & Curtis, 1987) and *Mycobacterium tuberculosis* (Phillip *et al.*, 1986). The other region of DNA which was sequenced, which lay

upstream from ORFB, did not show any significant homology to sequence in the databases searched and thus, was not analysed further in this study.

The homology described in this investigation between the 56 amino acid upstream of the *erm*-proximal end of transposition in mutant 9E and ArgC of other bacterial species was the first indication that such a protein existed in *L. monocytogenes*. Although attempts to confirm the presence of the remainder of the *arg*C gene at the *erm*-distal end of transposition were unsuccessful, the data strongly suggests that *arg*C had been disrupted in mutant 9E.

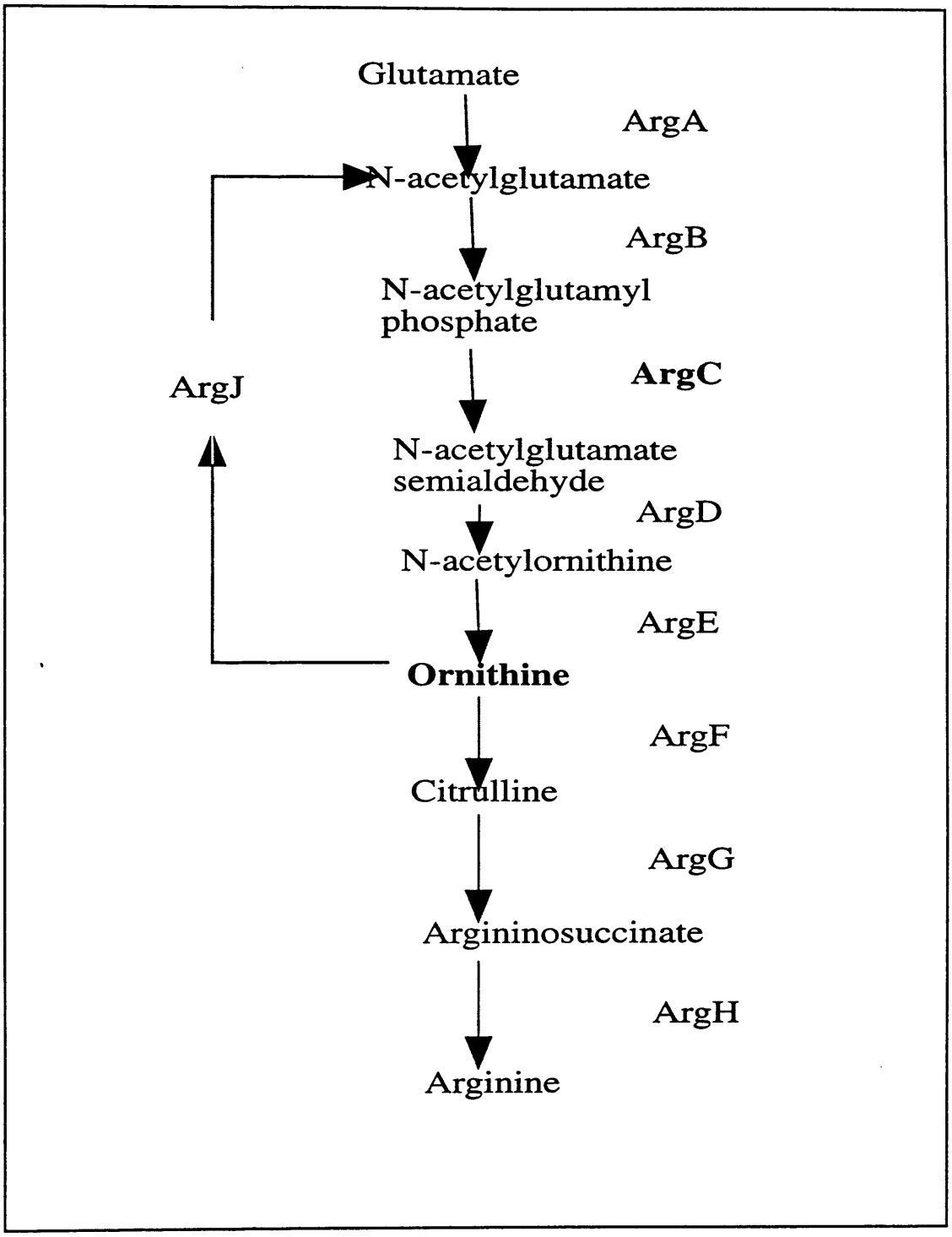
The putative promoter region of the listerial *arg*C gene was also analysed in this investigation. Consensus sequences for -10 and -35 were identified and interestingly an 18 bp motif with similarity to the consensus sequence for the *E. coli* Fur box (de Lorenzo *et al.*, 1988) was also identified upstream from the *arg*C gene. This suggested that expression of the *arg*C gene may by regulated by levels of environmental iron. Also identified in the *arg*C promoter region was a sequence similar to the Arginine box which has been reported upstream from *arg*C genes of numerous bacterial species including *B. su*btilis (O'Reilly & Devine, 1994), *Campylobacter jejuni* (Hani & Chan, 1994) and *E. coli* (Parsot *et al.*, 1988). These arginine boxes are involved in the arginine-regulated expression of *arg*C genes. The presence of arginine in the environment leads to the repression of these genes, thus the absence of β-galactosidase activity in mutant 9E may be explained by the fact that arginine in the medium was repressing activity from the *arg*C promoter which was present upstream from *lac*Z gene. To investigate this, the *argC* promoter could be cloned into a plasmid upstream from a promoterless copy of the *lacZ* and transformed into any bacterial strain which did not require arginine for growth. Measuring the activity of β-galactosidase expressed from the *argC* promoter in cells grown with and without arginine would determine if the listerial *argC* promoter region was inhibited by arginine. The measurement of β-galactosidase activity expressed from mutant 9E

grown in the absence of arginine could not be performed because *L. monocytogenes* could not grow in the absence of arginine (Trivett & Meyer, 1971).

To speculate on a relationship of the *arg*C mutation in mutant 9E to the inability of the mutant to grow in low-iron medium it is important to consider the function of ArgC in other bacterial species. The *arg*C gene encodes an enzyme, N-acetylglutamate semialdehyde dehydrogenase, which is involved in the arginine biosynthetic pathway (Cunin *et al.*, 1986). This enzyme catalyses the conversion of N-acetylglutamyl phosphate to N-acetylglutamate semialdehyde (Figure 4.2). The initial conclusion on discovering that a gene whose product is involved in the synthesis of arginine, was that an auxotrophic mutant had been identified. Arginine auxotrophs of *B. subtilis* (O'Reilly *et al.*, 1994), *Corynebacterium glutamicum* (Sakanyan *et al.*, 1996) and *Anabaena* species (Floriano *et al.*, 1992) have been reported and all have a characteristic growth dependency of arginine. However, the fact that this mutant was unable to grow in Trivett & Meyer's medium, which contained arginine suggested that some other factor was limiting the growth and it appeared to be iron levels. Mutant 9E was unable to grow in Trivett & Meyer's medium with reduced levels of ferrous iron (TM.Fe^{2+}) or ferric iron (TM.Fe^{3+}) but was able to grow in Trivett & Meyer's medium (N.TM) to which more iron was added. The only difference between these media was the levels of iron, thus, it was concluded that mutant 9E was altered such that it was unable to grow in low-iron environments, compared with the wild-type.

Interestingly, an intermediate of the arginine pathway, ornithine (Figure 4.2), was previously reported to be important for the synthesis of iron chelating compounds in other bacterial species (Cunin *et al.*, 1986; Emery, 1971). Ornithine or ornithine derivatives were shown to be important structural components of siderophores of numerous bacteria including, pyoverdin of *Pseudomonas aeruginosa* (Visca *et al.*,

Figure 4.2. Arginine biosynthetic pathway. A representation of the synthesis of arginine from glutamate, highlighting the role of the ArgC protein, N-acetylglutamate semialdehyde dehydrogenase.



Adapted from Cunin et al., (1986)

1994), exochelin of *Mycobacterium smegmatis* (Sharman *et al.*, 1995), Staphyloferrin A of *S. aureus* (Mewis et al., 1990), proferrioxamine of *Erwinia amylovora* (Feistner, 1995) and ornibactins of *Burkholderia cepacia* (Meyer *et al.*, 1995)

It is interesting to speculate that other, as yet unidentified compounds, involving ornithine or ornithine derivatives may be produced by *L. monocytogenes*. Although Cowart & Foster, (1985) examined *L. monocytogenes* for the production of siderophores and did not identify any it does not rule out the possibility of identifying other compounds with siderophore-like activity. These workers looked for both hydroxamate and phenolate siderophores in the culture supernate of *L. monocytogenes* grown under iron limitation using the methods of Csaky (1948) and Arnow (1937), respectively. None were identified. They also examined extracts of whole cells and still were unable to report the presence of siderphores. The failure of Cowart & Foster to identify siderophores in *L. monocytogenes*, may reflect the methods used to identify siderophores and also to create iron-limited conditions. These workers did not provide data which demonstrated the iron-limited conditions which were supposedly created by Chelex treatment of the medium being used. In addition, the assays of Csaky (1948) and Arnow (1937) identify siderophores which are structurally similar to hydroxamate or phenolate siderophores, respectively. They will not identify compounds which are structurally different from hydroxamate or phenolate siderophores but still have siderophore activity. Thus in future, experiments could be performed in the iron-limited medium used in this present study to examine the ability of culture supernates or cell extracts of *L. monocytogenes* to chelate iron, rather than looking specifically for siderophore molecules.

To determine if the inability of mutant 9E to grow in low-iron medium was related to the *argC* mutation and not the result of a point mutation or a polar effect on a gene downstream from *argC*, complementation of the mutation with the wild type gene could be performed. The results from this experiment would enable a conclusion to

be reached. Alternatively, supplementation of the low-iron medium with ornithine and examination of the growth would clarify the role of ornithine in iron acquisition of *L. monocytogenes*. Unfortunately a shortage of time prevented these areas from being further examined in the course of this study.

4.5 Concluding remarks

At the onset of this investigation, it was hoped that the *listerial* ferric reductase encoding gene(s) or other genes involved in the ability of *Listeria* to grow in low-iron medium *in vitro* would be identified. The investigation of the listerial ferric reductase was unsuccessful. However, two mutants were identified with an inability to grow in low-iron medium, *in vitro*.

Sequence analysis of the chromosomal DNA flanking transposition led to the conclusions that the *flaA* gene (Dons *et al.*, 1992) was disrupted in one mutant and a gene with homology to *argC* genes from other bacterial species (Smith *et al.*, 1990; Parsot *et al.*, 1988) was disrupted in the other mutant. The exact mechanism of how these mutations were resulting in an inability to grow in low-iron medium remains unclear.

It was speculated that the *flaA* mutation was causing the bacterium to become leaky and consequently allowing iron to diffuse out of the cytoplasm into the environment more rapidly than it would under normal conditions. This was supported by the increased levels of haemolytic activity also observed for this mutant. In the second mutant, it was likely that the *argC* mutation led to a disruption of the arginine biosynthetic pathway (Cunin *et al.*, 1986). One of the products of this pathway which would presumably not be produced was ornithine. Ornithine has been shown previously (Sharman *et al.*, 1995; Feistner, 1995; Meyer *et al.*, 1995) to be an important precursor for the synthesis of siderophores and siderophore-like molecules

in other bacterial species. Thus it was speculated here, that the presumed absence of ornithine production would result in an inability to synthesise siderophore-like molecules which consequently was resulting in an inability to grow in low-iron medium.

From the speculations made above, the conclusion was that both mutants, although phenotypically identical, with respect to their inability to grow in low-iron media, were producing this phenotype via different routes. It was proposed that the *flaA* mutation was resulting in an inability to grow in low-iron medium via a route which was not directly related to iron acquisition while the *argC* mutation was resulting in an inability to grow in low-iron medium due to disruption of a putative iron acquisition mechanism, involving ornithine. Unfortunately, within the time scale of this study, no experimental evidence was provided to support this proposition.

Previous investigations of other bacteria have reported the association of reduced virulence *in vivo*, with an inability to acquire iron *in vitro* (Montgomerie *et al.*, 1984; Cox, 1982). This study, reported the finding of two mutants with an inability to grow in low-iron *in vitro* but with no apparent affect on the growth of the bacteria *in vivo*; the reasons for this are unknown. It is possible that the mechanisms of *listerial* iron acquisition *in vivo* differ from those which occur *in vitro* The inability of both mutants to grow in low-iron medium *in vitro* would not have been of importance as the *in vivo* mechanisms of the mutants would be unaltered.

Future investigations of both these mutants may lead to clarification of the speculative propositions which have been made regarding their inability to grow in low-iron and the possible relationship (or not) of the mutation in each of these mutants to iron acquisition in *L. monocytogenes*.

REFERENCES

ADAMS, T. J., VARTIVARIAN, S., & COWART, E. (1990). "Iron acquisition systems of *Listeria monocytogenes*." *Infection and Immunity* **58**: 2715-2718.

ALEXANDER, J. E., ANDREW, P. W., JONES, D. & ROBERTS, I. S. (1993). "Characterisation of an aromatic amino acid-dependent *L. monocytogenes* mutant - attenuated virulence and ability to induce protective immune response." *Infection and Immunity* **61**: 2245-2248.

AMANN, E., J. BROSIUS, & M. PTASHNE. (1983). "Vectors carrying a hybrid trp-lac promoter useful for regulated expression of cloned genes in *Escherichia coli*." *Gene* **25**: 167-178.

AMARO, C., R. AZNAR, E. ALCAIDE, & M. L. LEMOS. (1990). "Iron-binding compounds and related outer membrane proteins in *Vibrio cholerae* non-O1 strains from aquatic environments." *Applied and Environmental Microbiology* **56**: 2410-2416.

ANDERSON, J. K. & MORRANG, B. (1995). Occurrence of L. monocytogenes in Danish foods. In, International Symposium on Problems of Listeriosis, Perth, WA, 1995.

Archibald, F. S. (1983). "*Lactobacillus plantarium*, an organism not requiring iron." *FEMS Microbiology Letters* **19**: 29-32.

ARCHIBALD, F. S. & I. W. DEVOE. (1979). "Removal of iron from human transferrin by Neisseria meningitidis." *FEMS Microbiology Letters* **6** : 159-162.

ARNOW, L. E. (1937). "Colorimetric determination of the components of 3, 4-dihydroxyphenylalanine-tyrosin mixtures." *Journal of Biological Chemistry* **118** : 531-537.

ATKINSON, E. (1917). "Meningitis associated with gram-positive bacilli of diptheroid type." *Medical Journal of Australia* **1** : 115-118.

AUDURIER, A., A. G. TAYLOR, B. CARBONELLE, & J. MCLAUGHLIN. (1984). "A phage typing system for *Listeria monocytogenes* and its use in epidemiological studies." *Clinical Investigative Medicine.* **7** : 229-232.

BAGG, A. & J. B. NEILANDS. (1987). "Molecular mechanisms of regulation of siderophore-mediated iron assimilation." *Microbiological Reviews* **51** : 509-518.

BAIG, B. H., . WACHSMUTH, & G. K. MORRIS. (1986). "Utilisation of exogenous siderophores by *Campylobacter* species." *Journal of Clinical Microbiology* **23** : 431-433.

BALDRIDGE, J. R., M. F. THOMASAW, & D. J. HINRICHS. (1988). "Induction of immunity with avirulent *Listeria monocytogenes* 19113 depends on bacterial replication." *Infection and Immunity.* **58** : 654.

BARRY, R. A., BOUWER, H. G., PORTNOY, D. A. & HINRICHS, D. J. (1992). "Pathogenicity and immunogenicity of *Listeria monocytogenes* small plaque mutant defective for intracellular growth and cell-to-cell spread." *Infection and Immunity* **60** : 1625-1632.

BEAMAN, L., & BEAMAN, B. L. (1984) "The role of oxygen and its derivatives in microbial pathogenesis and host defence." *Annual Review of Microbiology* **38** : 27-34.

BEATTIE, I. A., SWAMINATHAN, B., & ZIEGLE, H. G. (1990). "Cloning and characterisation of T-cell reactive protein antigen from *Listeria monocytogenes* and Listeria ivanovii." *Journal of Medical Microbiology* **30** : 119-123.

BELYI, Y. F., I. S. TARTAKOVSKII, & S. V. PROSOROVSKII. (1992) "Purification and characterisation of cell wall proteins of *Listeria monocytogenes.*" *Medical Microbiology and Immunology* **181** : 283-291.

BERCHE, P., J. L. GAILLARD, & P. J. SANSONETTI. (1987). "Intracellular growth of *Listeria monocytogenes* as a prerequisite for *in vivo* induction of T-cell mediated immunity." *Journal of Immunology*, **138** : 2266-2271.

BEUTLER, B & CERAMI, A. (1987). Cachectin tumour necrosis factor - a cytokine that mediates injury initiated by invasive parasites. *Parasitology Today* **3**: 345-346

BHATT, R., S. A. A. ALA'ALDEEN, T. BALDWIN, & S. P. BORRIELLO. (1994). "The 126 kDa iron-regulated protein of *Listeria*

monocytogenes is not a transferrin binding protein." *FEMS Microbiology Letters* **123** : 119-124.

BIELECKI, J., P. YOUNGMAN, P. CONNELLY, & D. A. PORTNOY. (1990). "*Bacillus subtilis* expressing a haemolysin gene from *Listeria monocytogenes* can grow in mammalian cells." *Nature* **345** : 175-176.

BISHOP, D. K., & HINRICHS, D. J. (1987). "Adoptive transfer of immunity to *Listeria monocytogenes*: the influence of *in vitro* stimulation on lymphocyte subset requirements." *Journal of Immunology* **143** : 2005.

BLEI, F. & D. R. PRUDER.(1993). "*Yersinia enterocolitica* bacteraemia in a chronically transfused patient with sickle cell anaemia." *Paediatric Haematology Oncology* **15** : 430-434.

BLENDEN, D. C., G. A. GATES, & S. L. SILBERG. "Epidemiological studies on an outbreak of listeriosis in a sheep flock." In Proceedings of the 3rd International Symposium on Listeriosis. in Bilthoven, Netherlands, , 6233-6241, Year.

BOHNE, J., Z. SOKOLIVIC, & W. GOEBEL. (1994). "Transcriptional regulation of *prfA* and *prfA*-regulated virulence genes in *Listeria monocytogenes*." *Molecular Microbiology* **11**: 1141-1150.

BORTOLUSSI, R., ISSEKUTZ, A., & FAULKNER, G. (1986). "Opsonisation of *Listeria monocytogenes* 4b by human adult and newborn sera." *Infection and Immunity* **52** : 493-498.

BRAUN, V., C. BRAZEL-FAISST, & R. SCHNEIDER. (1984). "Growth stimulation of *Escherichia coli* in serum by iron (III) enterobactin. Recycling of aerobactin." *FEMS Microbiology Letters* **21** : 99-103.

BREED, R. S., E. G. D. MURRAY, & A. P. HITCHENS.(1948). Bergey's Manual of Determinative Bacteriology. 6th ed., ed., Baltimore: Williams and Wilkins Co..

BREED, R. S., E. G. D. MURRAY, & N. R. SMITH. (1957). Bergey's Manual of Determinative Bacteriology. 7 th ed., Baltimore: Williams and Wilkins.

BREHM, K., HAAS, A., GOEBEL, W., & KREFT, J. (1992). "A gene encoding a superoxide dismutase of the facultative intracellular bacteria *Listeria monocytogenes*." *Gene* **118** : 121-125.

BROCK, J. H. & T. MAINOU-FOWLER. (1986). "Iron and immunity." *Proceedings of Nutritional Society* **45** : 305-315.

BROCK, J. H., P. H. WILLIAMS, P. H. LICEAGA, & K. G. WOOLDRIDGE. (1991). "Relative availability of transferrin-bound iron and cell-derived iron to aerobactin-producing and enterochelin-producing strains of *Escherichia coli* and to other microorganisms." *Infection and Immunity* **59** : 3185-3190.

BRUNT, L. M., D. A. PORTNOY, & E. R. UNANUE. (1990). "Presentation of *Listeria monocytogenes* to CD8+ T-cells requires secretion of haemolysin and intracellular bacterial growth." *Journal of Immunology* **145** : 3540-3546.

BUBERT, A., M. KUHN, W. GOEBEL, & S. KOHLER. (1992). "Structural and functional properties of the p60 proteins from different *Listeria* species." *Journal of Bacteriology* **174** (1992): 8166-8171.

BUCHANAN, R. E. & N. E. GIBBONS. (1974). Bergey's Manual of Determinative Bacteriology. 8th ed., Baltimore: Williams and Wilkins, 1974.

BULLEN, J. J., H. J. ROGERS, & E. GRIFFITHS. (1978). "Role of iron in bacterial infection." *Current Topics in Microbiology and Immunology* **80** : 1-35.

BULLEN, J. J. (1981). "The significance of iron in infection." *Review of Infectious Diseases* **3** : 1127-1138.

CAMILLI, A., D. A. PORTNOY, & P. YOUNGMAN. (1990). "Insertional mutagenesis of *Listeria monocytogenes* with a novel *Tn*917 derivative that allows direct cloning of DNA flanking transposon insertions." *Journal of Bacteriology* **172** : 3738-3744.

CAMILLI, A., H. GOLDFINE, & D. A. PORTNOY. (1991). "*Listeria monocytogenes* mutants lacking phosphatidylinositol specific phospholipase C are avirulent." *Journal of Experimental Medicine.* **173** : 751-754.

CAMILLI, A., L. G. TILNEY, & D. A. PORTNOY. (1993). "Dual role of *plcA* in *Listeria monocytogenes* pathogenesis." *Molecular Microbiology* **8** : 143-157.

CAMPBELL, J. (1990). Human listeriosis in Scotland 1967-1988. *Journal of Infectious Diseases.* **20**:241-250.

CARBONETTI, N. H., S. BOONCHAI, S. H. PARRY, & P. H. WILLIAMS. (1986). "Aerobactin-mediated uptake of iron by *Escherichia coli* isolates from human extraintestinal infections." *Infection and Immunity* **51** : 966-968.

CARDINEAU, G. A. & CURTISS, R. (1987). Nucleotide sequence of the *asd* gene of *Streptococcus mutans* - identification of the promoter region and evidence fro attenuator like sequences preceding the structural gene. *Journal of Biological Chemistry* **262**: 3344-3353.

CHAKRABORTY, T. & W. GOEBEL. (1988). "Recent developments in the study of virulence of *Listeria monocytogenes.*" Current Topics in Microbiology & Immunology **138** : 41-58.

CHART, H. & T. J. TRUST. (1983). "Acquisition of iron by *Aeromonas salmonicida.*" *Journal of Bacteriology* **156**: 758-764.

CHEN, Q., L. A. ACTIS, M. E. TOLMASKY, & J. H. CROSA. (1994). "Chromosome-mediated 2,3-dihydroxybenzoic acid is a precursor in the biosynthesis of the plasmid-mediated siderophore anguibactin in *Vibrio anguilarrium.*" *Journal of Bacteriology* **176** : 4226-4234.

CHEN, L. & J. D. HELMANN. (1994). "The *Bacillus subtilis* sigmaD-dependent operon encoding the flagellar proteins FliD, FliS and FliT." *Journal of Bacteriology* **176** : 3093-3101.

CHRISTIE, A., N. E. ATKINS, & E. MUNCH-PETERSON. (1944). "A note on a lytic phenomenon shown by group B Streptococci." *Australian Journal of Experimental Biology and Medical Sciences* **22** : 197-200.

CLARK & CARBON. (1977). A colony bank containing synthetic ColEI hybrid plasmid representative of the entire *E. coli* genome. *Cell* **9**: 91.

CLEWELL, D. B., S. E. FLANNAGAN, Y. IKE, J. M. JONES, & C. GAWRON-BURKE. (1988). "Sequence analysis of termini of conjugative transposon *Tn*916." *Journal of Bacteriology* **170** : 3046-3052.

COLLINS, M. D., S. FERESU, & D. JONES. (1983). "Cell wall DNA base composition, lipid studies of *Listeria denitrificans* (Prevot)." *FEMS Microbiology Letters.* **18** : 131-134.

COLLINS, M. D., S. WALLBANKS, D. J. LANE, J. SHAWN, R. NIETUPSKI, J. SMIDA, M. DORSCH, & E. STACKEBRANDT. (1991). "Phylogenetic analysis of the genus *Listeria* based on reverse-transcriptase sequencing of the 16S ribosomal RNA." *International Journal of Systematic Bacteriology.* **41** : 240-246.

COSSART, P. (1988). "The listeriolysin O-gene a chromosomal locus crucial for the virulence of *Listeria monocytogenes.*" *Infection* **16** : s2-s6.

COSSART, P., M. F. VICENTE, J. MENGAUD, F. BAQUERO, J. C. PEREZ-DIAZ, & P. BERCHE. (1989). "Listeriolysin O is essential for virulence of *Listeria monocytogenes*: direct evidence obtained by gene complementation." *Infection and Immunity.* **57** : 3629-3636.

COWART, R. E. & B. G. FOSTER. (1985). "Differential effects of iron on the growth of *Listeria monocytogenes*: minimum requirements and mechanism of acquisition." *Journal of Infectious Diseases.* **151**: 721-730 :

COWART, R. E. (1987). "Iron regulation of growth and haemolysin production by *Listeria monocytogenes.*" *Ann. Instit. Past. Microbiol.* **138** : 246-249.

COX, A. D. (1982). "Effect of pyochelin on the virulence of *Pseudomonas aeruginosa.*" *Infection and Immunity* **36** : 17-23.

COX, C. D. & P. ADAMS. (1985). "Siderophore activity of pyoverdin for *Pseudomonas aeruginosa.*" *Infection and Immunity* **46** : 130-138.

CROSA, J. H. (1984). "The relationship of plasmid-mediated iron transport and bacterial virulence." *Annual Reviews in Microbiology* **38** : 69-89.

CSAKY, T. Z. (1948). "On the estimation of bound hydroxylamine in biological materials." *Acta Chemical Scandinavia* **2** : 452-454.

CUNIN, R., N. GLANSDORFF, A. PIERARD, & V. STALON. (1986). "Biosynthesis and metabolism of arginine in bacteria." *Microbiological Reviews* **50** : 314-352.

DAILEY, H. A. & J. LASCELLES. (1977). "Reduction of iron and synthesis of protoheme by *Spirillum itersonii* and other organisms." *Journal of Bacteriology* **129** : 815-820.

DANCIS, A., R. D> KLAUSNER, A. G. HINNEBUSCH, & J. G. BARRIOCANAL. (1990). "Genetic evidence that ferric reductase is required for iron uptake in *Sacchromyces cerevisiae.*" *Molecular and Cellular Biology* **10** : 2294-2301.

DE LORENZO, V., F. GIOVANNINI, F. HERRERO, & J. B. NEILANDS. (1988). "metal iron regulation of gene expression. Fur-repressor-operator interaction at the promoter region of the aerobactin system of pColV-K30." *Journal of Molecular Biology* **203** : 875-884.

Deneer, H. G., (1995)

DENEER, H. G., & BOYCHUCK, I. (1993). "Reduction of ferric iron by *Listeria monocytogenes* and other species." *Canadian Journal of Microbiology* **39** : 480-485.

DENEER, H. G., HEALEY, V. & BOYCHUCK, I. (1995). Reduction of exogenous ferric iron by a surface associated ferric reductase of *Listeria* sppecies. *Microbiology* **141**:1986-1992.

DICK, G. F. (1920). "A case of cerebrospinal meningitis due to a diptheroid bacillus." *Journal of the American Medical Association.* **74** : 84.

DINARELLO, C. A. (1984). Interleukin-1 and the pathogenesis of the acute phase response. New England Journal of Medicine 311:1413-1418.

DOMANN, E., W. GOEBEL, M. LEIMEISTER-WACHTER, & T. CHAKRABORTY. (1991). "Molecular cloning. sequencing, and identification of a

metalloprotease gene from *Listeria monocytogenes* that is species specific and physically linked to the listeriolysin gene." *Infection and Immunity.* **59** : 65-72.

DONACHIE, W., & LOW, J. C. (1995). " Listeria monocytogenes: infection and immunity in animals." In, International Symposium on Problems of Listeriosis, Perth WA, 1995.

DONKER-VOET, J. (1965). "Listeriosis in animals." Bulletin de l'Office International des Epizooties. 64 : 757-764.

DONS, L., O. F. RASMUSSEN, & J. E. OLSEN. (1992). "Cloning and characterisation of a gene encoding flagellin of *Listeria monocytogenes.*" *Molecular Microbiology* **6** : 2919-2929.

DONS, L., J. E. OLSEN, & O. F. RASMUSSEN. (1994). "Characterisation of two putative *Listeria monocytogenes* genes encoding polypeptides homologous to the sensor protein CheA and the response regulator CheY of chemotaxis." *Journal of Sequencing and Mapping* **4** : 301-311.

DOUGAN, G., D. MASKELL, D. PICKARD, & C. HORMAECHE. (1987). "Isolation of stable aroA mutants of *Salmonella typhi* Ty2: properties and preliminary characterisation in mice." *Molecular and General Genetics.* **207**: 402-405.

DOWER, W. J., MILLER, J. F. & RAGSDALE, C. W. (1988). High efficiency transformation of Escherichia coli by high voltage electroporation. Nucleic Acid Research, 16: 6127-6145.

DRAMSI, S., C. KOCKS, C. FORESTIER, & P. COSSART. (1993). "Internalin-mediated invasion of epithelial cells by *Listeria monocytogenes* is regulated by the bacterial growth state, temperature and the pleiotropic activator PrfA." *Molecular Microbiology* **9** : 931-941.

DRAMSI, S., M. LEBRUN, & P. COSSART. (1994). "Molecular and genetic determinants involved in invasion of mammalian cells by *Listeria monocytogenes.*" *Current Topics in Microbiology* **192** : 61-77.

DRESCHEL, H., THIEKER, A., REISSBRODT, R., JANY, G.& WINKELMAN, G. (1993). Alpha ketoacids are novel siderophores in the genera

Proteus, Providencia and Morganella and are produced by amino acid deamination. *Journal of Bacteriology* **175**: 2727-2733.

DYVIG, K., HOLLINGSHEAD, S. K., HEATH, D. G., CLEWELL, D. B., SUN, F. & WOODARD, A. (1992). Degenerative oligonucleotide primers for enzymatic amplification of *recA* sequence from gram posistive bacteria nd mycoplasmas. *Journal of Bacteriology* **174**:2729-2732.

EMERY, T.(1971). "Hydroxamic acids of natural origin." In Advances in Enzymology, ed. A. Meister. 135-186. 35. New York: Whitley & Sons.

ESPAZE, E. P., A. F. MIEGEVILLE, & A.L. COURTIEU. "Morphologie et position taxonomique." In Proeceeding of the 9th International Symposium on the Problems of Listeriosis in Nantes, France, , 137-146, Year.

EVANS, S. L., J. E. L. ARCENEAUX, B. R. BYERS, M. E. MARTIN, & H. ARANHA. (1986). "Ferrous iron transport in *Streptococcus mutans*." *Journal of Bacteriology* **168** : 1096-1099.

FARBER, J. M. & PETERKIN, D. (1991). *Listeria monocytogenes*, a food borne pathogen. *Microbiological Reviews* **55**: 476-511.

FARBER, J. M., G. W. SANDERS, S. DUNFIELD, ¶ R. PRESCOTT.(1989). "The effect of various acidulants on the growth of *Listeria monocytogenes*." *Letters in Applied Microbiology.* **9**: 181-183.

FEISTNER, G. J. (1995). "Proferrioxamine synthesis in *Erwinia amylovora* in response to precursor or hydroxylysine feeding - metabolic profiling with liquid chromatography electrospray mass-spectrometry." *Biometal* **8** : 318-327.

FENLON, D. R. (1986). "Rapid quantitative assessment of the distribution of *Listeria* in silage implicated in a suspected outbreak of listeriosis in calves." *Veterinary Record.* **118** : 240-242.

FERESU, S. B. ¶ D. JONES. (1988). "Taxonomic studies on *Brochothrix, Erysipelothrix, Listeria* and atypical *Lactobacilli.*" *Journal of General Microbiology.* **5** : 360-376.

FIEDLER, F. ℑ J. SEGER. (1983). "The murein types of *Listeria grayi, Listeria murrayi,* and *Listeria denitrificans.*" *Systematic and Applied Microbiology.* **4** : 43-50.

FIEDLER, F., J. SEGER, A. SCHRETTENBRUNNER, & H. P. R. SEELIGER. (1984). "The biochemistry of murein and cell wall teichoic acids in the genus *Listeria.*" *Systematic and Applied Microbiology.* **5** : 360-376.

FIELD, L. H., V. L. HEADLY, S. M. PAYNE, & L. J. BERRY. (1986). "Influence of iron on growth, morphology, outer membrane composition, and synthesis of siderophores in *Campylobacter jejuni.*" *Infection and Immunity* **54**: 126-132.

FINLAY, B. B. Molecular genetic approaches to understanding bacterial pathogenesis.

FINLAY, B. B. & S. FALKOW.(1989). "Common themes in microbial pathogenicity." *Microbiological Reviews* **53** : 210-230.

FISCHETTI, V. A., PANCHOLI, V., SCHNEEWIND, O.(1990). "Conservation of a hexapeptide sequence in the anchor region of surface protein from Gram-positive cocci." *Molecular Microbiology* **4** : 1603-1605.

FLAMM, R. K., HINRICHS, D. J. & THAMSAW, H. (1984). Introduction of pAMB1 into *L. monocytogenes* by conjugatrion and homology between native *L. monocytogenes* plasmids. *Infection and Immunity* **44**:157-161.

FLEMING, D. W., S. I. COCHI, K. L. MACDONALD, J. BRODRUM, HAYES. P. S., B. D. PLIKARYTIS, M. B. HOLMES, A. AUDURIER, C. V. BROOME, & A. L. REINGOLD. (1985). "Pasteurised milk as a vehicle of infection in an outbreak of listeriosis." *New England Journal of Medicine.* **312** : 404-407.

FLORIANO, B., A. HERRERO, & E. FLORES. (1992). "Isolation of arginine auxotrophs, cloning by mutant complementation ,and sequence analysis of the *argC* gene from the cyanobacterium *Anabaena* species PCC 7120." *Molecular Microbiology* **6** : 2085-2094.

FREDRICK, K. L. & J. D. HELMANN. (1994). "Dual chemotaxis signalling pathways in *Bacillus subtilis*: a sigmaD-dependent gene encodes a novel protein with

both CheW and CheY homologous domains." *Journal of Bacteriology* **176**: 2727-2735.

FUZI, M. & I. PILLIS. (1962). "Production of opacity in egg yolk medium by *Listeria monocytogenes*." *Nature* **196** : 195.

GAILLARD, J. L., P. BERCHE, & P. SANSONETTI. (1986). "Transposon mutagenesis as a tool to study the role of haemolysis in the virulence of *Listeria monocytogenes*." *Infection and Immunity.* **52** : 50-55.

GAILLARD, J. L., P. BERCHE, J. MOUNIER, S. RICHARD, & P. SANSONETTI. (1987). "*In vitro* model of penetration and intracellular growth of *Listeria monocytogenes* in the human enterocyte-like cell line Caco-2." *Infection and Immunity.* **55** : 2822-2829.

GAILLARD, J-L., BERCHE, P., FREHEL, C., & COSSART, P. (1991). "Entry of *Listeria monocytogenes* into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from Gram-positive cocci." *Cell* **65** : 1127-1141.

GAINES, C. G., J. S. LODGE, J. E. L. ARCENEAUX, & B. R. BYERS. (1981). "Ferrisiderophore reductase activity associated with an aromatic biosynthetic enzyme complex in *Bacillus subtilis*." *Journal of Bacteriology* **148** : 527-533.

GALSWORTHY, S. B., GUROFSKY, S. F., & MURRAY, R. G. (1977). "Purification of a monocytosis-producing agent." *Infection and Immunity* **15** : 500-509.

GALSWORTHY, S. B. (1987). "Role of the cell surface in virulence of *Listeria monocytogenes*." *Annales de l'Institut Pasteur Microbiologie* **138**: 273-276.

GALSWORTHY, S. B., S. GIRDLER, & S. F. KOVAL.(1990). "Chemotaxis in *Listeria monocytogenes*." *Acta Microbiologica Hungarica* **37** : 81-85.

GELLIN, B. G. & C. V. BROOME.(1989). "Listeriosis." *Journal of the American Medical Association.* **261** : 1331-1321.

GENCO, C. A. & P. J. DESAI. (1996). "iron acquisition in the pathogenic *Neisseria*." *Trends in Microbiology* **4** : 179-184.

GEOFFROY, C., J. RAVENEAU, J. L. BERETTI, J. A. LECROISEY, J. VAZQUEZ-BOLAND, E. ALOUF, & P. BERCHE. (1991). "Purification and characterisation of an extracellular 25-kilodalton phospholipase C from *Listeria monocytogenes*." *Infection and Immunity* **59** 2382-2388.

GEORGATSOU, E. & D. ALEXANDRAKI. (1994). "Two distinctly regulated genes are required for ferric reduction, the first step of iron uptake in *Sacchromyces cerevisiae*." *Molecular and Cellular Biology* **14** : 3065-3073.

GIBSON, F. & D. I. MAGRATH. (1969). "The isolation and characterisation of a hydroxamic acid (aerobactin) formed by *Aerobacter aerogenes*." *Biochimie Biocphysics Acta* **192** : 174-184.

GILBERT, R. J., S. M. HALL, & A. G. TAYLOR. (1989). "Listeriosis update." *Public Health Laboratory Service Microbiology Digest.* **6** : 33-37.

GITTER, M. "Listeriosis in farm animals in Great Britain." In Symposium of the Society for Applied Bacteriology: Isolation and identification of microorganisms of medical and veterinary importance. in London, edited by Collins C. H. and J. M. Grang, Academic Press Inc., 191-200, Year.

GOEBEL, W., S. KATHARIOU, M. KUHN, Z. SOKOLOVIC, J. KREFT, S. KOHLER, D. FUNKE, T. CHAKRABORTY, & M. LEIMEISTER-WACHTER. (1988). "Haemolysin from Listeria - biochemistry, genetics, and function in pathogenesis." *Infection* **16** : 149-156.

GOEBEL, W., T. CHAKRABORTY, E. DOMANN, S. KOHLER, M. KHUN, M. LEIMEISTER-WACHTER, Z. SOKOLOVIC, 7 M. WUENSCHER. (1991). "Studies on the pathogenicity of *Listeria monocytogenes*." *Infection* **19** : s195-s197.

GOHMANN, S., LEIMEISTER-WACHTER, M., SCHLITZ, E., GOEBEL, W., & CHAKRABORTY, T. (1990). "Characterisation of a *Listeria monocytogenes*-specific protein capable of inducing delayed-hypersensitivity in mice." *Journal of Molecular Microbiology* **4** : 1091-1099.

GOLDFINE, H. & C. KNOB. (1992). "Purification and characterisation of *Listeria monocytogenes* phosphatidylinositol-specific phospholipase C." *Infection and Immunity* **60** : 4059-4067.

GOLDFINE, H., N. C. JOHNSTON, & C. KNOB. (1993). "non-specific phospholipase C of *Listeria monocytogenes*: activity on phospholipids in Triton X-100 -mixed micelles and in biological membranes." *Journal of Bacteriology* **175** : 4298-4306.

GOLDFINE, H., C. KNOB, D. ALFORD, & J. BENTZ. (1995). "Membrane permeabilisation of *Listeria monocytogenes* phosphatidylinositol-specific phospholipase C is independent of phospholipid hydrolysis and cooperative with listeriolysin O." *Proceedings of the National Academy of Science, USA* **92** : 2979-2983.

GONZALES, G. C., D. L. CAAMANO, & A. B. SCHRYVERS. (1990). "Identification and characterisation of a porcine-specific transferrin receptor in *Actinobacillus pleuropneumoniae*." *Molecular Microbiology* **4** : 1173-1179.

GOULET, V., REBERIE, I., & MARCHETTI, P. H. (1995). Listeriosis in France: Lessons from the investigation of this major national outbreak. In, International Symposium on Problems of Listeriosis, Perth WA, 1995.

GRAHAM, R., LEVINE, N. D., & MORRILL, C. C. (1943). "Listerellosis in domestic animals." *Agriculture Experimental Station Bulletin* **499** : 47-85.

GRAY, M. L. (1960). "Isolation of *Listeria monocytogenes* from oat silage." *Science* **132** : 1767-1768.

GRAY, M. L. 7 A. H. KILLINGER. (1966). "*Listeria monocytogenes* and listeric infections." *Bacteriological Reviews.* **30** : 309-382.

GRIFFITHS, G. L., S. P. SIGEL, S PAYNE M., & J. B. NEILANDS. (1984). "Vibriobactin, a siderophore from *Vibrio cholerae*." *Journal of Biological Chemistry.* **259** : 383-385.

GRIFFITHS, E., P. STEVENSON, T. L. HALE, & S. B. FORMAL. (1985). "Synthesis of aerobactin and a 76000-dalton iron-regulated outer membrane

protein by *Escherichia coli* K-12-*Shigella flexneri* hybrids and by enteroinvasive strains of *E. coli.*" *Infection and Immunity* **49** : 67-71.

GRIFFITHS, E. (1991). "Environmental regulation of bacterial virulence - implications fro vaccine design and production." *Trends in Biotechnology* **8** (1991): 309-315.

GRIFFITHS, E., CHART, H., & STEVENSON, P. (1992) "High affinity iron uptake systems and bacterial virulence." In Mechanisms of Bacterial Virulence, ed. J. A. Roth. 121-131.

GRIFFITHS, E. (1993). "Iron in infection: better understanding at the molecular level but little progress in the clinical front." *Journal of Medical Microbiology* **38** : 389-390.

GRIGGS, D. W. & J. KONISKY. (1989). "Mechanism for iron-regulated transcription of the *Escherichia coli cir* gene: metal -dependent binding of Fur protein to promoters." *Journal of Bacteriology* **171** : 1048-1054.

GUTIERREZ, J. A., P. J. CROWLEY, D. P. BROWN, J. D. HILLMAN, P. YOUNGMAN, & A. S. BLEIWEIS. (1996). "Insertional mutagenesis and recovery of interrupted genes of *Streptococcus mutans* by using transposon *Tn917: preliminary* characterisation of mutants displaying acid sensitivity and nutritional requirements." *Journal of Bacteriology* **178** (1996): 4166-4175.

GUTTERIDGE, J. M. C. (1987). "A method for removal of trace iron contamination from biological buffers." *FEBS Letters* **214** : 362-364.

GUZZO, A., C. DIORIO, & M. S. DUBOW. (1991). "Transcription of the *Escherichia coli fliC* gene is regulated by metal ions." *Applied and Environmental Microbiology* **57** : 2255-2259.

HAAS, A., BREHM, K., KREFT, J. & GOEBEL, W. (1991). "Cloning, characterisation, and expression in *Escherichia coli* of a gene encoding *Listeria seeligeri* catalase, a bacterial enzyme highly homologous to mammalian catalases." *Journal of Bacteriology* **173** : 5159-5167.

HAAS, A., & GOEBEL, W. (1991). "Cloning of a superoxide dismutase gene from Listeria ivanovii by functional complementation in *Escherichia coli* and characterisation of the gene product." *Molecular and General Genetics* **231** : 313-322.

HAHN, H., & KAUFMAN, S. H. E. (1981). "The role of cell mediated immunity in bacterial infections." *Review of Infectious Diseases* **3** : 1121-1250.

HALL, R. M., M. STRITHARAN, J. M. MESSENGER, & C. RATLEDGE. (1987). "iron transport in *Mycobacterium smegmatis*: occurrence of iron-regulated envelope proteins as potential receptors for iron uptake." *Journal of General Microbiology* **133** : 2107-2114.

HANAHAN, D. (1983). Studies on transformation of Escherichia coli with plasmids. Journal of Molecular Biology 166: 557.

HANI, E. K. & V. L. CHAN. (1994). "Cloning, characterisation and nucleotide sequence analysis of the argH gene from *Campylobacter jejuni* TGH9011 encoding argininosuccinate lyase." *Journal of Bacteriology* **176** : 1865-1871.

HANTKE, K. (1987). "Ferrous iron transport mutants in *Escherichia coli* K12." *FEMS Microbiology Letters* **44** : 53-57.

HARTFORD, T., S. O'BRIEN, P. W. ANDREW, D. JONES, & I. S ROBERTS. (1993). "Utilisation of transferrin-bound iron by *Listeria monocytogenes*." *FEMS Microbiology Letters* **108** : 311-318.

HENNECKE, H. (1990). "Regulation of bacterial gene expression by metal-protein complexes." *Molecular Microbiology* **4** : 1621-1628.

HENRY, B. S. (1933). Dissociation in the genera Brucella. *Jounral of Infectious Diseases* **52**: 374-402.

HEUCK, D., W. BEER, & R. REISSBRODT. (1995). "Iron supply of staphylococci and of micrococci by alpha-ketoacids." *Journal of Medical Microbiology* **43** : 26-32.

HOF, H., & CHATZIPANAGIOTOU, S. (1987). "The role of surface structures of *Listeria* species for pathogenicity." *Annales de l'Institut Pasteur Microbiologie* **138** : 268-273.

HOF, H. & P. HEFNER. (1988). "Pathogenicity of *Listeria monocytogenes* in comparison to other Listeria species." *Infection* **16** : 141-144.

HUYER, M. & W. J. PAGE. (1989). "Ferric reductase activity in *Azotobacter vinelandii* and its inhibition by Zn2+." *Journal of Bacteriology* **171** : 4031-4037.

ISSEKUTZ, T. B., EVANS, J., & BORTULUSSI, K. (1984). "The immune response of human neonates to *Listeria monocytogenes* infection." *Clinical and Investigative Medicine* **7** : 281-286.

IVANOV, I., M. DRAGNOV, & T. DIKOVA. "Study of the active immunoprophylaxis of sheep listeriosis." In Proceedings of the VIIth International Symposium of Listeriosis in Sofia, Bulgaria, Year.

IVANOV, I. "Results of the administration of the live attenuated vaccine listeriosis." In Proceedings of the VIIIth International Symposium of Listeriosis. in Madrid, Spain, Year.

JACOBSON, S. H., K. TULLUS, B. WRETLIND, & A. BRAUNER. (1988). "Aerobactin-mediated uptake of iron by strains of *Escherichia coli* causing acute pyelonephritis and bactetaemia." *Journal of Infection.* **16** : 147-152.

JACQUES, C., CATAMEL, B., GOULET, V., LEPOUTRE, A., VEIT, P., DEHAUMONT, P. & ROCOURT, J. (1995). Typing of L. monocytogenes during epidemiological investigation of French listeriosis outbreaks in 1992, 3 and 5. In, International Symposium on Problems of Listeriosis, Perth, WA, 1995.

JOHNSON, W., L. VARNER, & M. POCH. (1991). "Acquisition of iron by *Legionella pneumophila*: role of iron reductase." *Infection and Immunity* **59** : 2376-2381.

JONES, D. (1975). "The taxonomic position of Listeria." In Problems of Listeriosis, ed. M. Woodbine. 14-17. Leicester: Leicester University Press.

JONES, D., S. B. FERESU, & M. D. COLLINS. "Classification and identification of Listeria, Brochothrix, and Erysipelothrix." In Proceedings of the 9th

International Symposium on the Problems of Listeriosis in Nantes, edited by A. L. Courtieu, E. P. Espaze, and A. E. Reynaud, Year.

JONES, S. & D. A. PORTNOY. (1994). "Characterisation of *Listeria monocytogenes* pathogenesis in a strain expressing perfringolysin O in place of listeriolysin O." *Infection and Immunity* **62** : 5608-5613.

JONES, C. E., G. SHAMA, P. W. ANDREW, I. S. ROBERTS, & D. JONES. (1995). "Comparative study of the growth of *Listeria monocytogenes* in defined media and demonstration of growth in continuous culture." *Journal of Applied Bacteriology* **78**: 66-70.

KATHARIOU, S., P. METZ, H. HOF, & W. GOEBEL. (1987). "*Tn*916-induced mutations in the haemolysin determinant affecting virulence of *Listeria monocytogenes*." *Journal of Bacteriology.* **80** : 683-692.

KATHARIOU, S., P. METZ, H. HOF, & W. GOEBEL. (1988). "Levels of *Listeria monocytogenes* haemolysin are not directly proportional to virulence in experimental infections in mice." *Infection and Immunity.* **56** (1988): 534-536.

KATHARIOU, S., L. PINE, V. GEORGE, G. M. CARLOS, & B. P. HOLOWAY. (1990). "Nonhaemolytic *Listeria moncytogenes* mutants that are noninvasive for mammalian cells in culture; evidence for coordinate regulation of virulence." *Infection and Immunity.* **58** : 3988-3995.

KATHARIOU, S., R. KANENAKA, R. D. ALLEN, A. K. FOK, & C. MIZUMOTO. (1995). "Repression of motility and flagellin production at 37oC is stronger in *Listeria monocytogenes* than in the nonpathogenic species Listeria innocua." *Canadian Journal of Microbiology* **41** : 572-577.

KAUFMANN, S. H. E., HAHN, H., & KIRCHNER, H. (1983). "Interferon-gamma produced by *Listeria monocytogenes* specific T-cells active in cellular anti-bacterial immunity." *European Journal of Immunology* **3**: 265-268.

KAUFMANN, S. H. E. (1984). "Acquired resistance to facultative intracellular bacteria: relationship between persistence, cross-reactivity at the T-cell level, and capacity to stimulate cellular immunity of different Listeria strains." *Infection and Immunity* **45** : 234-241.

KEARNS, S. & D. HINRICHS. (1977). "Kinetics and maintenance of acquired resistance in mice to *Listeria monocytogenes.*" *Infection and Immunity* . **16** : 923-927.

KESSLER, M., B. HOEN, D. MAYEUX, D. HESTIN, & C FONTENAILLE. (1993). "Bacteraemia in patients on chronic hemodialysis." *Nephron* **64** : 95-100.

KINGSLEY, R., W. RABSCH, M. ROBERTS, R. REISSBRODT, & P. H. WILLIAMS. (1996). "TonB-dependent iron supply in *Salmonella* by alpha-ketoacids and alpha-hydroxyacids." *FEMS Microbiology Letters* **140** : 65-70.

KOCKS, C., MARCHAND, J.B., GOUIN, E., DHAUTEVILLE, H., SANSONETTI, P. J., CARLIER, M. F. & COSSART, P. (1995). The unrelated surface-proteins actA of Listeria monocytogenes and icsA of Shigella flexneri are sufficient to confer actin based motility on listeria innocua and escherichia coli respectively. Molecular Microbiology, 18:413-423.

KOHLER, S., S. LEIMEISTER-WACHTER, T. CHAKRABORTY, F. LOTTSPEICH, & W. GOEBEL. (1990). "The gene encoding for protein p60 of *Listeria monocytogenes* and its use as a specific probe for *Listeria monocytogenes.*" *infection and Immunity.* **58** : 1943-1950.

KOJIMA, N. & G. W. BATES. (1973). "The reduction and release of iron from Fe3+-transferrin-Co32-." *Journal of Biological Chemistry* **254** : 8847-8854.

KONETSCHNY-RAPP, S., S. JUNG, J. MEIWES, 7 H. ZAHNER. (1990). "Staphyloferrin A: a structurally new siderophore from staphylococci." *European Journal of Biochemistry* **191** : 65-74.

KUHN, M. & W. GOEBEL. (1989). "Identification of an extracellular protein of *Listeria monocytogenes* possible involved in intracellular uptake by mammalian cells." *Infection and Immunity.* **57** : 55-61.

KUHN, M., D. T. PREVOST, W. T. MELVIN, & R. POSTLETHWAITE. (1990). "A nonvirulent mutant of *Listeria monocytogenes* does not move intracellularly but still induces polymerisation of actin." *Infection and Immunity.* **58**: 3477-3486 :

LAEMMLI, U. K. (1970). "Cleavage of structural proteins during assemble of the head of bacteriophage T4." *Nature* **227** (1970): 680-685.

LEBLOND-FRANCILLARD, M., GAILLARD, J-L., & BERCHE, P. (1989). "Loss of catalase in *Tn*1545-induced mutants does not reduce growth of *Listeria monocytogenes in vivo*." *Infection and Immunity* **57**: 2569-2573.

LEIMEISTER-WACHTER, M, E. DOMANN, & T. CHAKRABORTY. (1991). "Detection of a gene encoding a phosphatidylinositol specific phospholipase C that is co-ordinately expressed with listeriolysin in *Listeria monocytogenes*." *Molecular Microbiology*. **5** : 361-366.

LINDE, K., A. A. ABRAHAM, & J. BEER. (1991). "Stable *Listeria monocytogenes* live vaccine candidate strains with graded attenuation on the mouse model." *Vaccine* **9** : 101-105.

LINMAN, M. J., L. MASCOLA, S. D. LOU, V. GOULET, S. MAY, D. SALMINEN, W. HIRD, M. L. YONEKURA, P. HAYES, R. WEAVER, AUDURIER. A., B. D. PLIKARYTIS, S. L. FANNIN, S. KELKS, & C. V. BROOME. (1988). "Epidemic listeriosis associated with Mexican-style cheese." *New England Journal of Medicine*. **319** : 823-834.

LITWIN, C. M. & B. CALDERWOOD. (1993). "Role of iron in regulation of virulence genes." *Clinical Microbiology Reviews* **6** : 137-149.

LOW, M. G. (1990.). "Degradation ofglycosyl - phosphatidylinositol anchors of by specific phospholipases." In Molecular and cell biology of membrane proteins: glycolipid anchors of cell surface proteins., ed. A. J. Turner. 35-63. Chichester, England: Howard, E. Ltd.

LUI, P. V. & J. L. BATES. (1961). "An extracellular haemorrhagic toxin produced by *Listeria monocytogenes*." *Canadian Journal of Microbiology* **7** : 107-108.

LUND, B. M. (1990) "The prevention of foodborne listeriosis." *British Food Journal*. **92**:13-22.

MACKANESS, G. B. (1969). "The influence of immunologically committed lymphoid cells on macrophage activity in vivo." *Journal of Experimental Medicine* **129** : 973-992.

MACNAB, R. M. (1987). "Motility and chemotaxis." In *Escherichia coli and Salmonella typhimurium*: Cellular and Molecular Biology., ed. F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger. 732-359. Washington D. C.: American Society of Microbiology.

MACNAB, R. M. (1996). 'Structural Flaegllin Synthesis'. *Escherichia coli and Salmonella typhimurium*: Cellular and Molecular Biology., ed. F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger. 732-359. Washington D. C.: American Society of Microbiology.

MACRINA, F. L. (1984). "Molecular cloning of bacterial antigens and virulence determinants." *Annual Reviews in Microbiology* : 193-219.

MALINVERNI, R., J. BILLE, C. PERRET, F. REGLI, F. TANNER, & M. P. GLAUSER. (1985). "Listeriose epidemique." *Schweizerische Medizinische Wochenschrift.* **115** : 277-282.

MARCELIS, J. H., H. J. DEN DAAS-SLAGT, & J. A. A. HOOGKAMP-KORSTANJE. (1978). "Iron requirement and chelator production of Staphylococci, Streptococcus faecalis and Enterobactericae." *Antonie van Leewenhoek Journal of Microbiology* **44** : 257-267.

MARKWELL, M. A. K., S. M. HAAS, L.L. BIEBER, & N. E. TOLBERT. (1978). "A modification of the Lowry procedure to simplify protein determination in membrane samples." *Analytical Biochemistry* **87** : 206-210.

MARQUIS, H., V. DOSHI, & D. A. PORTNOY. (1995). "The broad-range phospholipase C and a metalloprotease mediate listeriolysin O-dependent escape of *Listeria monocytogenes* from a primary vacuole in human epithelial cells." *Infection and Immunity* **63** : 4531-4534.

MARTINEZ, J. L., E. CERCENADO, F. BAQUERO, J. C. PEREZ-DIAZ, & A. DELGADO-IRIBARREN. (1987). "Incidence of aerobactin production in Gram-negative hospital isolates." *FEMS Microbiology Letters* **43** : 351-353.

MAURELLI, A. T. (1989). "Temperature regulation of virulence genes in pathogenic bacteria: a general strategy for human pathogens?" *Microbial Pathogenesis* **7** : 1-10.

MCCARTER, L. & M. SILVERMANN. (1990). Surface-induced swarmer cell differentiation of *Vibrio parahaemolyticus. Molecular Microbiology* : 1057-1062.

MCLAUGHLIN, J. (1987). "A review. *Listeria monocytogenes*, recent advances in the taxonomy and epidemiology of listeriosis in humans." *Journal of Bacteriology.* **63** : 1-11.

MCLAUGHLIN, J. & NEWTON, C. (1995). Human listeriosis in England, Wales and Northern Ireland, a changing pattern of infection. In, International symposium on Problems of Listeriosis, Perth, WA, 1995.

MEKALANOS, J. J. (1992). "Environmental signals controlling expression of virulence determinants in bacteria." *Journal of Bacteriology* **174**: 1-7.

MENGUAD, J., C. BRAUNBRETE, & P. COSSART. (1991). "Identification of phosphatidylinositol specific phospholipase C activity in *Listeria monocytogenes* - a novel type of virulence factor." *Molecular Microbiology.* **5** : 367-392.

MEYER, J. M., V. T. VAN, A. STINTZI, O. BERGE, & G. WINKELMANN. (1995). "Ornibactin production and transport - properties in strains of *Burkholderia vietnamiensis* and *Burkholderia cepacia* (formerly *Pseudomonas cepacia*)." *Biometals* **8** : 309-317.

MICHEL, E., K. A. REICH, R. FAVIER, P. BERCHE, & P. COSSART. (1990). "Attenuated mutants of the intracellular bacterium *Listeria monocytogenes* obtained by single amino acid substitutions in listeriolysin O." *Molecular Microbiology.* **4** : 2167-2178.

MILES, A. A. & P. L. KHIMJI. (1975). "Enterobacterial chelators of iron: their occurrence, detection and relation to pathogenicity." *Journal of Medical Microbiology* **8** : 477-492.

MILLER, J. H. (1972). Experiments in molecular genetics. Cold Spring Harbour: Cold Spring Harbour Laboratory.

MONNO, R., M. A. VALENZA, M. QUARTO, V. SABATO, D. DE MATTIA, G. PARADIES, L. MONTINARO, & D. FUMAROLA. (1994). "*Yersinia enterocolitica* infection in a boy with b-thalassemia major." *Paediatric Infectious Disease Journal* **13** : 233-234.

MONTGOMERIE, J. A., A. BINDEREIF, J. B. NEILANDS, G. M. KALMANSON, & L. B. GUZE. (1984). "Association of hydroxamate siderophore (aerobactin) with *Escherichia coli* isolated from patients with bacteraemia." *Infection and Immunity* **46** : 835-838.

MORTON, D. J., & WILLIAMS, P. (1989). "Utilisation of transferrin bound iron by Haemophilus species of human and porcine origins." *FEMS Microbiology Letters* **65** : 123-128.

MOUNIER, J., A. RYTER, M. COQUIS-RONDON, & P. J. SANSONETTI. (1990). "Intracellular and cell-to-cell spread of *Listeria monocytogenes* involves interaction with F-actin in the enterocyte-like cell line Caco-2." *Infection and Immunity* **58** : 1048-1058.

MUKKUR, T. J. K., B. A. D. STOCKER, & K. H. WALKER. (1991). "Genetic manipulation of *Salmonella* serotype Bovis morbificant to aromatic-dependence and evaluation of its vaccine potential in mice." *Journal of General Microbiology.* **34** : 57-63.

MURPHY, J. R., A. M. PAPPENHEIMER, & S. TAYART DE BORMS. (1978). "Evidence that the regulation of diptheria toxin production is directed at the level of transcription." *Journal of Bacteriology* **135** : 511-516.

MURRAY, E. G. D., R. A. WEBB, & M. B. R. SWANN. (1926). "A disease of rabbits characterised by a large mononuclear leucocytosis, caused by a hitherto undescribed bacillus Bacterium monocytogenes (n. sp.)." *Journal of Pathological Biology* **29**: 407-439.

NASSIF, X. & P. J. SANSONETTI. (1986). "Correlation of the virulence of Klebsiella pneumoniae K1 and K2 with the presence of a plasmid encoding aerobactin." *Infection and Immunity* **54** : 603-608.

NAVARRE, W. W. & O. SCHNEEWIND. (1994). "Proteolytic cleavage and cell was anchoring at the LPXTGX motif of surface proteins in Gram-positive bacteria." *Molecular Microbiology* **14** : 115-121.

NEIBUHR, K., T. CHAKRABORTY, P. KOLLNER, & J. WEHLAND. (1993). "Production of monoclonal antibodies to the phosphatidylcholine-specific phospholipase C of *Listeria monocytogenes*, a virulence factor for this species." *Medical Micriobiology Letters* **2** : 9-16.

NEILANDS, J. B. (1981). "Iron absorption and transport in microorganisms." *Annual Reviews in Biochemistry* **50** : 715-731.

NJOKI-OBI, A. N., E. M. JENKINS, C. NJOKI-OBI, J. ADAMS, & V. COVINGTON. (1963). "Production and nature of *Listeria monocytogenes* haemolysins." *Journal of Bacteriology.* **86** : 1-8.

NORROD, P., & WILLIAMS, P. (1978). "Growth of *Neisseria gonorrhoeae* in media deficient in iron without detection of siderophores." *Current Microbiology* **7** : 281-284.

NOTERMANS, S. H. W., J. DUFRENNE, M. LEIMEISTER-WACHTER, E. DOMANN, & T. CHAKRABORTY. (1991). "Phosphatidylinositol specific phospholipase C activity as a marker to distinguish pathogenic and nonpathogenic Listeria species." *Applied and Environmental Microbiology.* **57** : 2666-2670.

O'CALLAGHAN, D., D. MASKELL, F. Y. LEIW, C. S. F. EASMON, AND G. DOUGAN. (1988). "Characterisation of aromatic and purine-dependent *Salmonella typhimurium*: attenuation, persistence and ability to induce protective immunity in BALB/c mice." *Infection and Immunity.* **56** : 419-423.

O'REILLY, M. & K. M. DEVINE. (1994). "Sequence and analysis of the citrulline biosynthetic operon *arg*C-F from *Bacillus subtilis*." *Microbiology* **140** : 1023-1025.

O'REILLY, M., K. WOODSON, B. C. DOWDS, & K. M. DEVINE. (1994). "The citrulline biosynthetic operon, *arg*C-F, and a ribose transport operon, rbs, from *Bacillus subtilis* are negatively regulated by SpoOA." *Molecular Microbiology* **11** : 87-98.

ODEGAARD, B., R. GRELLAND, & S. D. HENRICKSON. (1952). "A case of listeric infection in man transmitted from sheep." *Acta. Med. Scandin.* **67** : 231-238.

OGUNNARIWO, J. A., ALCANTARA, J., & SCHRYVERS, A. B. (1991). "Evidence for non-siderophore mediated acquisition of transferrin-bound iron by *Pasteurella multocida.*" *Microbial Pathogenesis* **11** : 47-56.

OTTO, B. R., VERWIJ-VAN-VUGHT, A., M., J., & J., MACLAREN, D., M. (1992). "Transferrins and heme-compounds as iron sources for pathogenic bacteria." *Critical Review in Microbiology* **18**: 217-233.

OWEN, C. R., A. MEISS, J. W. JACKSON, & H. G. STOEMER. (1960). "A case of primary cutaneous listeriosis." *New England Journal of Medicine.* **262** : 1026-1028.

PARRISIUS, J., S. BHAKDI, M. ROTH, J. TRANUM-JENSEN, W. GOEBEL, & H. P. R. SEELIGER. (1986). "Production of listeriolysin by beta-haemolytic strains of *Listeria monocytogenes.*" *Infection and Immunity.* **51** : 314-319.

PARSOT, C., A. BOYEN, G. N. COHEN, & N. GLANSDORFF. (1988). "Nucleotide sequence of *Escherichia coli argB* and *argC* gene: comparison of N-acetylglutamate kinase and N-acetylglutamate-gamma-semialdehyde dehydrogenase with homologous and analogous enzymes." *Gene* **68**: 275-283.

PAYNE, S. M. (1988). "Iron and virulence in the family *Enterobacteriaceae.*" *Critical Reviews in Microbiology* **16** : 81-111.

PERRY, R. D. & R. R. BRUBAKER. (1979). "Accumulation of iron by *Yersinae.*" *Journal of Bacteriology* **137** : 1290-1298.

PHILLIP ET AL. (1996). *Proceedings for the National Academy of Science, USA* **93** : 3132-3137.

PIDOCK, K. A., J. A. WOOTEN, B. A. DALEY, & T. L. STULL. (1988). "Iron acquisition by *Haemophilus influezae.*" *Infection and Immunity* **56**: 721-725.

PIRIE, J. H. H. (1927). "A new disease of veldt rodents "Tiger River Disease"." *Publication of the South African Institute of Research.* **2** : 163-185.

POCH, M. T. & W. JOHNSON. (1993). "Ferric reductases of *Legionella pneumophila.*" *Biometals* **6** : 107-114.

POLLOCK, J. R. 7 J. B. NEILANDS. (1970). "Enterobactin, an iron transport compound from *Salmonella typhimurium.*" *Biochemistry and Biophysics Research Communication* **38** : 989-992.

POPE, C. D., W.A. O'CONNELL, 7 N. P. CIANCIOTTO. (1996). "*Legionella pneumophila* mutants that are defective fro iron acquisition and assimilation and intracellular infection." *Infection and Immunity* **64** : 629-636.

PORTNOY, □D. A., P. S. JACKS, & D. J. HINRICHS. (1988). "Role of haemolysin fro the intracellular growth of *Listeria monocytogenes.*" *Journal of Experimental Medicine.* **167**: 1459-1471.

PORTNOY, D. A., T. CHAKRABORTY, W. GOEBEL, & P. COSSART. (1992). "Molecular determinants of *Listeria monocytogenes* pathogenesis." *Infection and Immunity.* **60** : 1263-1267.

POYART, C., E. ABACHIN, I. RAZAFIMANANTSOA, & P. BERCHE. (1993). "The zinc metalloprotease of *Listeria monocytogenes* is required for maturation of phosphatidylcholine phospholipase C: direct evidence obtained by gene complementation." *Infection and Immunity* **61** : 1576-1580.

POYART, C., TRIEUCUOT, P. & BERCHE, P. (1996). The *inlA* gene required for cell invasion is conserved and specific to *Listeria monocytogenes. Microbiology, UK* **142**: 173-180

RACZ, P., K. TENNER, & E. MERO. (1972). "Experimental Listeria enteritis. An electron microscopic study of the epithelial phase in experimental infection." *Laboratory Investigation* **26** : 694-700.

RALEIGH, E. A. & WILSON, G. (1986). *Escherichia coli* K-12 restricts DNA containing 5-methylcytosine. *Proceeding for the National Academy of Science USA* **83**: 9070.

RALOVICH, B. Listeriosis Research - Present Situation and Perspective. Budapest: Akadiai Kiado, 1984.

RAVENEAU, J., C. GEOFFROY, J. L. BERRETI, J. L. GAILLARD, J. E. ALOUF, 7 P. BERCHE. (1992). "Reduced virulence of a *Listeria monocytogenes* phospholipase-deficient mutant obtained by transposon insertion into the zinc metalloprotease gene." *Infection and Immunity.* **60** : 916-921.

REDHEAD, H., T. HILL, & H. CHART. (1987). "Interaction of lactoferrin and transferrins with the outer membrane of *Bordetella pertussis.*" *Journal of General Microbiology* **133** : 891-898.

RICARD, M. A., ARCHIBALD, F. S., &NIVEN, D. F. (1991). "Isolation and identification of a putative porcine transferrin receptor from *Actinobacillus pleuropneumoniae* biotype 1." *Journal of General Microbiology* **137** : 2733-2740.

ROCOURT, J., G. GRIMONT, P. A. D. GRIMONT, & H. P. R. SEELIGER. (1982). "DNA relatedness among serovars of *Listeria monocytogenes.*" *Current Microbiology.* **7** : 383.

ROCOURT, J., J.M. ALONSO, & H. P. R. SEELIGER. (1983). "Comparative virulence toward the five genomic groups of *Listeria monocytogenes.*" *Annales Institute Pasteur Microbiology* **134** : 359-364.

ROCOURT, J., U. WEHMEYER, & E. STACKEBRANDT. (1987a). "Transfer of *Listeria denitrificans* to a new genus *Jonesia* gen. nov. as *Jonesia denitrificans* comb. nov." *International Journal of Systematic Bacteriology.* **37** : 317-330.

ROCOURT, J., U. WEHMEYER, P. COSSART, & E. STACKEBRANDT. (1987b). "Proposal to retain *Listeria murrayi* and *Listeria grayi* in the genus *Listeria.*" *International Journal of Systematic Bacteriology.* **37** : 298-300.

ROCOURT, J., P. BOERLIN, F. GRIMONT, C. JACQUET, & J. C. PIFARETTI. (1992). "Assignment of *Listeria grayi* and *Listeria murrayi* to a single species, *Listeria grayi*, with a revised description of *Listeria grayi.*" *International Journal of Systematic Bacteriology.* **42** : 171-174.

ROGERS, H. J., C. SYNGE, C. KIMBER, & P. M. BAYLEY. (1977). "Production of enterochelin by *Escherichia coli* 0111." *Biochimie Biophysics Acta* **497** : 548-557.

ROQUETTE, C., J. M. BOLLA, & P. BERCHE. (1995). "An iron-dependent mutant of *Listeria monocytogenes* of attenuated virulence." *FEMS Microbiology Letters* **133** : 77-83.

ROSARIO, M. M. L., K. L. FREDRICK, G. W. ORDAL, & J. D. HELMANN. (1994). "Chemotaxis in *Bacillus subtilis* requires either of two functionally redundant CheW homologs." *Journal of Bacteriology* **176** : 2736-2739.

RYSER, E. T. & E. H. MARTH. (1991). "*Listeria monocytogenes*: Characteristics and Classification." In *Listeria* and Listeriosis and Food Safety, 1-22. New York: Marcel Dekker Inc., 1991.

SAKANYAN, V., P. PETROSYAN, M. LECOCQ, A. BOYEN, C. LEGRAIN, M. DEMAREZ, J. N. HALLET, & N. GLANSDORFF. (1996). "Genes and enzymes of the acetyl cycle of arginine biosynthesis in *Corynebacterium glutamicum: enzyme* evolution in the early steps of the arginine pathway." *Microbiology* **142** (1996): 99-108.

SAMBROOK, J., FRITSCH, E. F. & MANIATIS, T. (1989). Molecular cloning: A laboratory manual, 2nd edition. Cold Spring Harbour, Laboratory Press, Cold SPring Harbour, NY.

SCHLECH, W. F., P. M. LAVIGNE, & R. BORTOLUSSI. (1983). "Epidemic listeriosis: Evidence for transmission by food." *New England Journal of Medicine.* **308** : 203-206.

SCHLEIFER, K. H. & O. KANDLER. (1971). "Peptidoglycan types of bacterial cell walls and their taxonomic implications." *Bacteriology Reviews.* **36** : 407-477.

SCHRYVERS, A. B. & L. J. MORRIS. (1988). "Identification and characterisation of the human lactoferrin-binding protein from *Neisseria meningitidis.*" *Infection and Immunity* **56** : 1144-1149.

SCHRYVERS, A. B. (1989). "Identification of the transferrin and lactoferrin binding proteins in *Haemophilus influenzae*." *journal of Medical Microbiology* **29** : 121-130.

SCHRYVERS, A. B. & B. C. LEE. (1989). "Comparative analysis of the transferrin and lactoferrin binding proteins in the family *Neisseriaceae*." *Canadian Journal of Microbiology* **35** : 409-415.

SEELIGER, H. P. R. (1961). Listeriosis. New York.: Hafner Publishing Co.,, 1961.

SEELIGER, H. P. R. & K. HOHNE. (1979). "Serotyping of *Listeria monocytogenes* and related species." In *Methods in Microbiology*, ed. T. Bergen and J. R. Norris. 31-49. 13. Orlando, Florida: Academic Press Inc, 1979.

SEELIGER, H. P.R. & D. JONES. (1986). Listeriosis. 9th ed., ed., Bergey's manual of Systematic Bacteriology, ed. J. G. Holt. Baltimore,: Williams and Wilkins, 1986.

SHARMAN, G. J., D. H. WILLIAMS, D. F. EWING, & C. RATLEDGE. (1995). "Isolation, purification and structure of exochelin MS, the extracellular siderophore from *Mycobacterium smegmatis*." *biochemical Journal* **305** : 187-196.

SHAW, J. H. & D. B. CLEWELL. (1985) "Complete nucleotide sequence of macrolide-lincosamide-streptomigrin B-resistance transposon *Tn*917 in *Streptococcus faecalis*." *Journal of Bacteriology* **164**: 782-796.

SHEENAN, B., C. KOCKS, S. DRAMSI, E. GOUIN, A. D. KLARSFELD, J. MENGAUD, & P. COSSART. (1994). "Molecular and genetic determinants of *Listeria monocytogenes* infectious process." *Current Topics in Microbiology* **192** : 187-216.

SHORT, JM., FERNANDEZ, J. M., SORGE, J. A. & HUSE, W. D. (1988). Lambda ZAP; a bacteriophage lambda expression system with *in vivo* excision properties. *Nucleic Acid Research*, **16**: 7583.

SHUM, D. T., & GALSWORTHY, S. B. (1982). "Stimulation of monocyte production by an endogenous mediator induced by a component from *Listeria monocytogenes*." *Immunology* **46** : 343-349.

SIGEL, S. P., J. A. STOEBNER, & S. M. PAYNE. (1985). "Iron-vibriobactin transport system is not required fro virulence of *Vibrio cholerae*." *Infection and Immunity* **28** : 360-362.

SIMON, N., V. CONLANGES, P. ANDRE, & D. J. M. VIDON. (1995). "Utilisation of exogenous siderophores and natural chatecols by *Listeria monocytogenes*." *Applied and Environmental Microbiology*, **61** : 1643-1645.

SKALKA, B., J. SMOLA, & K. ELISSCHEROVA. (1982). "Routine test for in vitro differentiation of pathogenic and apathogenic *Listeria monocytogenes*." *Journal of Clinical Microbiology* **15** : 503-507.

SMITH, M. C. M., A. MOUNTAIN, & S. BAUMBERG. (1986). "Sequence analysis of the *Bacillus subtilis argC* promoter region." *Gene* **49** : 53-60.

SMITH, M. C. M., A. MOUNTAIN, & S. BAUMBERG. (1990) "Nucleotide sequence of the *Bacillus subtilis argC* gene encoding N-acetylglutamate-gamma-semialdehyde dehydrogenase." *Nucleic Acid Research* **18**: 4595.

SMITH, G. A., H. MARQUIS, S. JONES, N. C. JOHNSTON, & D. A. PORTNOY. (1995). "The two distinct phospholipases C of *Listeria monocytogenes* have overlapping roles in escape from a vacoule and cell-to-cell spread." *Infection and Immunity* **63** : 4231-4237.

SOKOL, P. A. & D. E. WOODS. (1983). "Demonstration of an iron-siderophore binding protein in the outer membrane of *Pseudomonas aeruginosa*." *Infection and Immunity* **55** : 2021-2025.

SOKOL, P.A. (1987). "Tn5 insertion mutants of *Pseudomonas aeruginosa* deficient in surface expression of ferripyochelin-binding protein." *Journal of Bacteriology* **169** : 3365-3368.

SOUTHERN, E. M. (1975). Detection of specific sequences among DNA fragment separated by gel electrophoresis. *Journal of Molecular Biology* **98**: 503-519.

STARK, M. J. R. (1987). "Multicopy expression vectors carrying the lac repressor gene for regulated high-level expression of genes in *Escherichia coli.*" *Gene* **51** : 255-267.

STEPHENS, J. C., I. S. ROBERTS, D. JONES, & P. W. ANDREW. (1991). "Effect of growth temperature on virulence of strains of *Listeria monocytogenes* in the mouse:evidence for a dose dependence." *Journal of Applied Bacteriology* **70** : 239-244.

STOEBNER J. A. & S. M. PAYNE. (1988). "Iron-regulated haemolysin production and utilisation of haeme and haemoglobin by *Vibrio cholerae.*" *Infection and Immunity* **56** : 2891-2895.

STOJILJKOVIC, I., A. J. BAUMLER, & K. HANTKE. (1994) "Fur regulon in Gram-negative bacteria. Identification and characterisation of new iron-regulated *Escherichia coli* genes by a Fur titration assay." *Journal of Molecular Biology* **236** : 531-535.

STOOKEY, L. L. (1970). "Ferrozine: a new spectrophotometric reagent for iron." *Analytical Chemistry* **42** : 779-781.

STRITHARAN, M. & C. RATLEDGE. (!988). "Coordinated expression of the components of iron transport (mycobactin, exochelin and envelope proteins) in *Mycobacterium neoaurum.*" *FEMS Microbiology Letters* **60** : 183-186.

STUART, M. E. & P. E. PEASE. (1972). "A numerical study on the relationships of Listeria and Erysipelothrix." *Journal of General Microbiology.* **73**: 551-565.

STUART, S. E. & H.J. WELSHIMER. (1973). "Intrageneric relatedness of Listeria Pirie." *International Journal of Systematic Bacteriology.* **23** : 177-185.

STUART, S. E. & H. J. WELSHIMER. (1974). "Taxonomic reexamination of Listeria Pirie and transfer of *Listeria grayi* and *Listeria murrayi* to a new genus, Murraya." *International Journal of Systematic Bacteriology.* **24** : 177-185.

SUN, A. N., A. CAMILLI, & D. A. PORTNOY. (1990). "Isolation of *Listeria monocytogenes* small-plaque mutants defective fro intracellular growth and cell-to-cell spread." *Infection and Immunity.* **58** : 3770-3778.

SWORD, C. P. (1966). "Mechanisms of pathogenesis in Listeria monocytogenes infection: influence of iron." *Journal of Bacteriology* **92** : 536-542.

TAI, S. S. & R. K. HOLMES. (1988). "Iron regulation of the cloned diphtheria toxin promoter in *Escherichia coli.*" *Infection and Immunity* **56** : 2430-2436.

TAI, S. S., C. C. LEE, & R. E. WINTER. (1993). "Hemin utilisation is related to virulence of *Streptococcus pneumoniae.*" *Infection and Immunity* **61** : 5401-5405.

TRIVETT, T. L. & E. A. MEYER. (1971). "Citrate cycle and related metabolism of *Listeria monocytogenes.*" *Journal of Bacteriology* **107** : 770-779.

VADILLO, M., X. CORBELLA, P. VISITACION, P. FERNANDEZ-VILADRICH, & R. PUJOL. (1994. "Multiple liver abscesses due to *Yersinia enterocolitica* discloses primary hemochromatosis: three case reports and review." *Clinical Infectious Diseases* **18**): 938-941.

VASCONCELOS, J. A. P. & DENEER, H. G. (1994). Expression of superoxide dismutase in *Listeria monocytogenes.* Applied *and environmental Microbiology* **60**: 2360-2366.

VAZQUEZ-BOLAND, J., C. KOCKS, S. DRAMSI, H. OHAYON, C. GEOFFROY, J. MENGAUD, & P. COSSART. (1992). "Nucleotide sequence of the lecithinase operon of *Listeria monocytogenes* and possible role of lecithinase in cell-to-cell spread." *Infection and Immunity.* **60** 219-230.

VIDON, D. J. M. & M. SPRENG. (1992)"Effect of some iron-chelating agents on growth of *Listeria monocytogenes.*" In 11th International Symposium on Problems of Listeriosis in Copenhagen, , 340-341, Year.

VISCA, P., A. CIERVO, & N. ORSI. (1994). "Cloning and nucleotide sequence of the pvdA gene encoding the pyoverdin biosynthetic enzyme L-ornithine N-oxygenase in *Pseudomonas aeruginosa.*" *Journal of Bacteriology* **176** : 1128-1140.

WEINBERG, E. D. (1978). "Iron and infection." *Microbiological Reviews* **42** : 45-66.

WEINBERG, E. D. (1984). "Iron withholding: a defence against infection and neoplasia." *Physiological Reviews* **64** : 65-102.

WEINBERG, E. D. (1995). "Acquisition of iron and other nutrients in vivo." In Virulence mechanisms of bacterial pathogens., ed. J. Roth and C. A. Bolin. 81-95. Washington D. C.: American Society for Microbiology, 1995.

WEINBERG, E. D. & G. A. WEINBERG. (1995). "The role of iron in infection." *Current opinion in Infectious Diseases* **8** : 164-169.

WELCH, D. F., SWORD. C. P., BREHM, S., & DUNSANIC, D. (1979). "Relationship between superoxide dismutase and pathogenic mechanisms of *Listeria monocytogenes.*" *Infection and Immunity* **23**: 863-872.

WELCH, D. F. (1987). "Role of catalase and superoxide dismutase in the virulence of *Listeria monocytogenes.*" *Annales de l'Institut Pasteur Microbiolgie* **138** : 265-276.

WELSHIMER, H. J. (1963). "Vitamin requirements of *Listeria monocytogenes.*" Journal *of Bacteriology* **85** : 1156-1159.

WELSHIMER, H. J. (1981). "The genus Listeria and related organisms." In The Prokaryotes. A handbook on habitats, isolation and identification of bacteria., ed. Starr et. al. 1680-1687. New York: Springer, 1981.

WERTMAN, K. F., WYMAN, A. R., BOTSTEIN, D. (1986). Host vector interactions which affect the viability of recombinant phage lambda clones. *Gene* **49**:253-262.

WILKINSON, B. J. & D. JONES. (1977). "A numerical taxonomic survey of *Listeria* and related bacteria." *Journal of General Microbiology.* **98** : 399-421.

WILLIAMS, R. P. (1990). In Iron Transport and Storage, ed. P. Ponka Schilman, H. P., & Woodwoth, C. R. C. 2-15. Cleveland: 1990.

WILLIAMS, P., D. J. MORTON, K. J. TOWNER, P STEVENSON, & E. GRIFFITHS. (1990). "Utilisation of enterobactin and other exogenous iron sources by *Haemophilus influenzae, H. parainfluenzae and H. paraprophilus.*" *Journal of General Microbiology* **136** : 2343-2350.

WILLIAMS, P., & GRIFFITHS, E. (1992). "Bacterial transferrin receptors: structure, function and contribution to virulence." *Medical Microbiology and Immunology* **181** : 301-322.

WIRSING VON KOENIG, C. H. & H. FINGER. (1982). "Failure of killed *Listeria monocytogenes* vaccine to produce protective immunity." *Nature* **297**: 233-234.

WIRSING VON KOENIG, C. H., B. HEYMER, & H. HOF FINGER, H. (1983). "Course of infection and development of immunity in experimental infection of mice with *Listeria* serotypes." *Infection and Immunity.* **40** : 1170-1177.

WOHLER, W. H. & C. L. BAUGH. (1983). "Pulmonary listeriosis in feeder cattle." *Modern Veterinary Practise* **64** : 736-739.

WOOLDRIDGE, K. G. & P. H. WILLIAMS. (1993). "Iron uptake mechanisms of pathogenic bacteria." *FEMS Microbiology Reviews* **12** : 325-348.

WUENSCHER, M., S. KOHLER, A. BUBERT, U. GERIKE, & W. GOEBEL. (1993). "The iap gene of *Listeria monocytogenes* is essential for cell viability and its gene product, p60, has bacteriolytic activity." *Journal of Bacteriology* **175** : 3491-3501.

YANISCH-PERON, C., VIERIA, J., & MESSING, J. (1985). Improved vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**:103.

YOUNGMAN, P. J., J. B. PERKINS, & R. LOSICK. (1983). "Genetic transposition and insertional mutagenesis." *Proceedings of the National Academy of Science, USA* **80**: 2305-2309.

YOUNGMAN, P. (1987). Plasmid vectors for recovering and exploiting *Tn*917 transpositions in *Bacillus* and other Gram-positive bacteria. Plasmids, a Practical Approach, ed. K. G. Hardy. Oxford, UK: IRL Press, 1987.

YOUNGMAN, P. J. (1990). "Use of transposon and insertional mutagenesis inn Bacillus subtilis with *Streptococcus faecalis* transposon *Tn*917." In Molecular biology methods for Bacillus., ed. C. R. Harwood and S. M. Cutting. 221-266. New York: 1990.